A STUDY OF THE ANTIGENS OF FOOT-AND-MOUTH DISEASE VIRUS BY COMPLEMENT FIXATION

by

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ABSTRACT

A study was made of the fixation of complement by antigens of foot-and-mouth disease virus (FMDV). The technique was applied to the determination of the serological properties of FMDV antigens and to the differentiation and classification of strains.

An examination was made of the relationships between antigen, antibody and the fixation of complement. Complement fixation (CF) with FMDV followed the same principles as models developed in other antigen/antibody systems. The assumption that there is a relationship of direct proportionality between the amount of complement fixed and the amount of antiserum reacting with constant antigen, was found to be incorrect. An alternative method was proposed for the quantitative differentiation of FMDV strains.

Applying this method, isolated FMDV antigens were examined for type and sub-type specificity. The intact virion was type specific and possessed considerable sub-type specificity. In contrast, capsid sub-units reacted heterotypically and with less sub-type specificity. A non-structural virus infection-associated antigen had virtually no type or sub-type specificity. Empty capsids were shown to possess the same sub-type specificity as intact virions, while trypsin-treated virus was more cross-reactive. The implications of the serological activity of these antigens were discussed, in relation to the structure of the virion and to the application of CF to routine typing and sub-typing procedures.

The CF test was compared to cross-protection and cross-

neutralisation tests for the determination of differences between strains and found to produce similar results. A large number of strains of one type were compared in cross-CF tests and from the results it was shown that the present methods of sub-type classification are unsatisfactory. A new system for the sub-type classification of FMDV strains was proposed.

TABLE OF CONTENTS

ERRATA

Page 39, line 25 should read "... between the amount of antiserum and the amount ..."

Page 71, line 3 should read "This antiserum was used..."

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DECLARATION

Part of the work presented in this thesis has been accepted for publication. The references are as follows:

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GENERAL INTRODUCTION

Foot-and-mouth disease (FMD) is an acute and highly contagious infection of ruminants and swine. It is characterised by the development of vesicles, particularly in the oral cavity and the interdigital and coronary skin. While it is generally not a killing disease except in young animals, the acute stage of the disease is often followed by a prolonged period of convalescence which results in a marked loss of production of meat and milk and of reproductive performance.

The disease is endemic throughout most of Europe, Asia, Africa and South America and has made incursions into most other parts of the world. It has never occurred in New Zealand and probably only once in Australia in 1872 (Seddon, 1966). It last occurred in the United States in 1929, in Canada in 1952 and in Mexico in 1953 (Callis et al., 1968). Britain maintains an uneasy freedom, having suffered its ravages almost anually to 1968 when the last epidemic was halted. Control of the disease during the 1967-68 epidemic resulted in the slaughter of more than 400,000 livestock at a direct cost of over £35 million (Ministry of Agriculture, 1968).

FMD is tolerated only at the cost of inefficient livestock production. It is controlled by expensive vaccination schemes and by the regulation of livestock movement. Freedom is maintained only by stringent quarantine regulations. Thus no country is unaffected by the disease which remains one of the most economically important diseases of livestock.

FMD was the first animal disease demonstrated to be of viral aetiology when in 1897, Loeffler and Frosch showed that

the causative agent passed through bacterial filters. Foot-and-mouth disease virus (FMDV) was later shown to possess certain characteristics which enabled the International Committee for Nomenclature of Viruses to classify it in the Picornaviridae family (Wildy, 1971). The members are small (20-30 nm.), isometric (probably icosanedral), naked vertebrate viruses, ether-resistant and containing single-stranded RNA. The Committee further classified FMDV into the genus Rhinovirus, mainly on the basis of its instability at pH3. However, Newman et al. (1973) have questioned the validity of this grouping and have suggested an alternative classification of picornaviruses into six sub-groups on the basis of bouyant density in caesium chloride, stability over the range of pH3 to 7 and the base composition of the RNA genome. Their system places FMDV in a sub-group with equine rhinoviruses but not with human rhinoviruses.

FMDV was the first virus shown to possess immunological variability when in 1922, Vallée and Carré demonstrated on the basis of cross-immunity tests, two distinct types, O and A. Waldmann and Trautwein (1926) distinguished a further type, C, and three others were isolated from Southern African Territories; types SAT 1, SAT 2, and SAT 3 (Brooksby, 1958). Finally, a seventh type, Asia 1, was discovered in 1954 (Brooksby and Rogers, 1957).

While the seven FMDV types are immunologically quite distinct, within each type there is a range of antigenic variation between strains. Such variation was first demonstrated by Waldmann and Trautwein (1926) by cross-immunity tests and by Bedson et al. (1927) in cross serum neutralisation

tests. Strains within a type have been classified into groups designated as sub-types. The significance of such classification is primarily in relation to the protection of immunised animals against field challenge with a different strain.

In 1946, Traub and Möhlmann first applied complement-fixation (CF) to the differentiation of intra-type strains and since then, this method has become universally popular for the identification of sub-types. However, many other immunological methods have also been used.

Cross serum neutralisation tests have been used by various workers (Brooksby et al., 1948 b; Hyslop et al., 1963; Hedger and Herniman, 1966) to demonstrate antigenic differences between strains. However the methods used have generally given poor reproducibility and have not found wide application.

An agar gel precipitation test has been described by Graves (1960) for FMDV sub-type differentiation. However, difficulties of accurate measurement of the reactions preclude the technique from use for quantitative studies. The radial immunodiffusion test of Mancini et al. (1964; 1965) has a greater potential but has not been used for the differentiation of FMDV strains. It has however, been applied by Cowan and Wagner (1970) for detection and measurement of FMDV antibodies with considerable success although it was found to have limitations, in that reversed diffusion (antiserum placed in the wells and antigen incorporated into the gel) was less satisfactory for the accurate measurement of antibody levels.

Other methods which have been applied include a passive haemagglutination inhibition test (Warrington and Kawakami, 1972)

and a passive immuno-haemolysis test (Wittmann and Reda, 1972). Both of these methods were used to demonstrate sub-type differences and were reproducible, but have not been applied extensively.

The CF test is one of the most universally applied methods for the detection and quantification of serological reactions. The methods used in most situations are based on standard techniques (see for example Casey, 1965) which are valid in terms of the criteria determined by earlier workers for the quantitative estimation of the fixation of complement.

However the method which has been used in the World Reference Laboratory for FMD at this Institute, and elsewhere, for the demonstration of sub-type differences with FMDV is unique and differs in the assumptions on which it is based. The only experimental justification for the method (Bradish et al., 1960 a) is open to interpretation and is contradictory to the criteria determined with other complement-fixing systems.

The first Section of this thesis describes experiments carried out to investigate the relationships between antigen, antibody and the fixation of complement in the FMDV system. Evidence is presented suggesting that the basis of the method described by Bradish et al. (1960 a) is invalid and a different approach is proposed for the differentiation of FMDV strains by CF.

Considerable knowledge of the structure of FMDV has been gained from biochemical, biophysical and serological studies with the virus. However, in the main, serological investigations of antigenic components of FMDV have been approached from a qualitative point of view and there is very little quantitative

information regarding the type and sub-type specificities of viral components. It has been demonstrated that the antigenic activity of intact virus particles is type specific (Brown and Crick, 1958) but cross-reactive between sub-types (Bradish and Brooksby, 1960). However, cross-reactions between types occur with 12S particles (Brown and Crick, 1958; Bradish and Brooksby, 1960), which are the structural sub-units of the viral capsid. A non-structural viral antigen, identified by Cowan and Graves (1966) (virus infection-associated antigen), also reacts heterotypically. Some studies of the type specificity of separated FMDV components were carried out by Bradish and Brooksby (1960). However, the more recent work of Cowan and Graves (1966), has revealed a greater antigenic complexity of FMDV infected tissues and tissue-culture harvests than was appreciated at the time and this limits the value of their findings.

The second section of this work presents studies by CF of the serological specificity of isolated antigenic components of the virus. The importance of the heterogeneity of naturally-occurring viral antigens used in routine CF tests for typing and sub-typing is assessed, and the implications of the results are considered in relation to current knowledge of the structure of the virus.

Antigenic differentiation of strains has been used in an attempt to classify intra-type variants into sub-type groups. However the difficulty in attempting to classify into discrete groups, strains whose degrees of difference are apparently continuously variable has led to ambiguity and confusion. The most important uses of strain differentiation have been

epidemiological, particularly in their application to the control of FMD in the field; notably in the monitoring of endemic situations for the early detection of emergent or introduced new sub-types and in the comparison of field strains with those incorporated into vaccines. The use of CF tests for such purposes can be criticised on the grounds that serological differences so determined may not reflect the antigenic differences that determine whether or not an animal is protected against field challenge.

This criticism is indeed pertinent, as there is virtually no satisfactory quantitative evidence for a correlation between differences detected by CF and those manifest by *in vitro* neutralisation tests or by animal cross-protection tests. Some such evidence is provided in the third section of this thesis, together with an evaluation of the use of CF for sub-type classification of FMDV strains.

SECTION 1. THE FIXATION OF COMPLEMENT BY MIXTURES OF FOOT-AND-MOUTH DISEASE VIRUS ANTIGEN AND ANTIBODY

1.1. INTRODUCTION

The use of CF for the quantitative measurement of reactions between antigen and antibody was pioneered by Wadsworth and his co-workers (Wadsworth *et al.*, 1923 a, b and c). The basis of their method was the finding that under certain conditions, with either antigen or antibody at a suitable fixed concentration, there was a linear relationship between the amount of complement fixed and the amount of the other reactant (antibody or antigen respectively.)

Further studies were made by Mayer and others. With a system of pneumococcal polysaccharide and homologous antibody, Mayer *et al.* (1948) and Osler *et al.* (1948) confirmed the findings of Wadsworth's team, but demonstrated clearly that, in antigen excess, the relationship between the amount of antiserum and the amount of complement fixed, although linear, was not directly proportional. However with antiserum in excess, there was a direct relationship between the amount of antigen and the amount of complement fixed.

Osler and Heidelberger (1948 a and b) studied homologous and heterologous reactions between pneumococcal type III and type VIII polysaccharide systems and between chicken- and duck-egg albumin systems. They showed that homologous and heterologous reactions could be differentiated by measuring the amount of complement fixed with a constant amount of one antiserum and an optimal amount of either homologous or

heterologous antigen. The homologous reaction was generally greater although the difference between two antigens was not necessarily apparent with both antisera.

Wallace et al. (1950), using a system of crystalline bovine serum albumin (BSA) and rabbit anti-BSA serum, studied the extent of complement-fixation as a function of the amount of antibody. When antibody was reacted with antigen at an optimal dilution (that giving maximum fixation of complement) the relationship of the amount of complement fixed to the amount of antibody could be described by a sigmoid curve. They concluded that in their system, the most satisfactory end-point determination of an antiserum titre was the estimation of the dilution of antiserum which would fix 50% of the complement available in the presence of the optimum antigen concentration.

From the criteria determined by these various workers, CF tests have been developed for many microbiological systems using a standard technique (e.g. Casey, 1965). The basis of the method is to test a series of reaction mixtures with varying antigen and antiserum dilutions in a so-called "chequerboard" titration, with a fixed amount of complement. From the results a maximum serum titre can be determined, being the highest dilution of antiserum fixing a defined amount of complement. The optimal antigen dilution can also be obtained, being that producing the maximum antiserum titre. Having determined the activities of standard antigens and antisera, unknown antigens can be titrated in known antiserum excess and unknown sera can be titrated with a known optimum dilution of antigen.

CF tests for the type differentiation of FMDV strains were described by Cuica (1929) and Traub and Möhlmann (1943). In 1946, Traub and Möhlmann showed that the test could be used to demonstrate variants within a type. Their test was based on the comparison of homologous and heterologous antiserum titres, determined as the highest dilution of a serum capable of fixing a defined amount of complement with undiluted vesicular extracts as antigen. Brooksby et al. (1948 a) demonstrated strain differences by comparing the ability of mixtures of undiluted antigen with homologous and heterologous antisera to fix complement, their results being expressed as the dose of complement required to produce 50% haemolysis.

Bradish et al. (1960 a) and Bradish and Brooksby (1960) used CF tests to study the antigenic components of FMDV and the specificity of their interactions with antiserum. They compared titrations of antigen in antiserum excess and antiserum in antigen excess for their ability to reveal differences in specificity. Finding the latter technique to be more sensitive, they carried out antiserum titrations and expressed their results as the amount of complement fixed per unit volume of undiluted antiserum, on the assumption that the amount of complement fixed was directly proportional to the amount of antiserum. Cross-fixation ratios were determined as the proportion of complement-fixing activity of the antigen in heterologous compared with homologous reactions. This method was adopted as the basis for sub-type differentiation in the World Reference Laboratory (see, for example: Davie, 1964; Darbyshire et al., 1972).

Roumiantzeff et al. (1965) described a method for the measurement of complement fixation by FMDV which was applied to the differentiation of strains. (Roumiantzeff et al., 1966). These workers used conventional chequerboard titrations of antigen and antiserum from which antiserum titres were derived. The titres of an antiserum with heterologous and homologous antigens were expressed as a proportion, to give a quantitative indication of the antigenic difference between the strains.

In this Section, a description is given of experiments carried out to determine the relationships between the amounts of FMDV antigen and antibody in a mixture and the amount of complement fixed. From the results, a different method is proposed for the differentiation of FMDV strains by CF and applications of the method are described using tubes and microplate CF tests.

1.2 MATERIALS AND METHODS

1.2 (a) Reagents

The preparation of veronal-buffered saline (VBS), complement and the sensitised erythrocyte suspension is described in Appendix 1.

The antigens used were 140S preparations, purified from virus harvests by the method described by Brown and Cartwright (1963). The methods of virus growth and purification are described in Appendix 2. The strains used were as follows:-

(i) Two type O strains, O_6 (OV1) and O_1 Lombardy (O_1Lom.). The numeral sub-scripts denote separate sub-type classifications

by the World Reference Laboratory;

(ii) Two type A strains, A6003 and A6900, which had been compared by CF previously by Guerche et al. (1972);

(iii) Three type Asia 1 strains, being a parent strain, Asia 1 C2, and two derived strains, Asia 1 387 and Asia 1 415, which had been selected under mutagenic conditions for altered electrophoretic mobility. The strains had earlier undergone extensive comparative studies (Priston, 1972). They were supplied by Dr. R. Priston as purified 140S antigens.

Antisera to the type O and type A strains were produced in guinea pigs following the method of Brooksby (1952) (Appendix 3 (a)). Antisera to the Asia 1 strains were provided by Dr. R. Priston. They were produced by inoculating guinea pigs once only with crude tissue culture harvests and bleeding them 28 days later. Before use the antisera were inactivated for 30 minutes at 56°C.

1.2 (b) Protocol of the Tests

In tube tests, the reaction mixtures were prepared in disposable spectrophotometer cuvettes (Walter Sarstedt (U.K.) Ltd., Leicester) of 1 cm. path length and 3.5 ml. capacity. Addition of all reagents was performed with the tubes in a water bath at 0°C, using fixed volume micro-pipettes (Eppendorf Geratebau Netheler u. Hinz GmbH, Hamburg, Germany). Antigen, complement and antiserum were added in that order, each in a volume of 500 µl. In control mixtures, reagents were replaced as appropriate by the addition of the same volume of VBS in the same order. The mixtures were then incubated in a water-bath for 30 minutes at 37°C and sensitised erythrocytes added in a volume of 1000 µl. After a further incubation for 45 minutes at 37°C, the tubes were chilled and centrifuged at 600 g. for 10 minutes to sediment unlysed cells.

The OD of each mixture was then measured at 541 nm. and the proportion of lysis calculated relative to that in control tubes showing 100% lysis (OD = 0.600 ± 0.010).

The amount of complement fixed in each reaction mixture showing lysis between 10% and 90%, was calculated using the formula of von Krogh (1916), viz.

$$\log x = \log k + (1/n) \log y/(1 - y),$$

where x = the amount of complement remaining after primary fixation,

k = the amount of complement required for 50% haemolysis ($1\ C'H_{50}$),

1/n = a constant and

y = proportion of erythrocytes lysed.

The value of 1/n was determined for the system by measuring the degree of haemolysis resulting from the addition of graded amounts of complement. It was established that 1/n varied between 0.18 and 0.22 and a constant value of 0.20 was used. The value of k was calculated using the same formula, from complement control mixtures where x was taken as the amount of complement added.

Microplate tests were performed in wells in plastic plates (Linbro - Biocult, Biocult Laboratories Ltd., Glasgow) in a final volume of 125 µl. The conditions used were designed as far as possible to parallel those used in tube tests. Thus, the reagents were added in the same order, all in 1/20 of the volume used in tubes (antigen, complement and antiserum, 25 µl.; sensitised erythrocytes, 50 µl.) All dilutions were made in bottles and the reagents added to the plates with calibrated dropping pipettes. Mixtures were incubated in a hot-air incubator for 60 minutes prior to the addition of sensitised erythrocytes, which was followed by a further incubation at 37°C for 45 minutes and then centrifugation

at 600 g. for 5 minutes to sediment unlysed cells. It was established by carrying out comparative tests in parallel, that primary fixation under these conditions was similar to fixation in tubes after 30 minutes in a waterbath at 37°C. The tests were read by visual estimation of the size of deposited erythrocyte buttons.

1.3 RESULTS

1.3 (a) A Model for the Fixation of Complement by Purified 140S Antigens and Antiserum

In order to elucidate the relationships of complement-fixation to the concentrations of antigen and antiserum, CF tests were carried out using a fixed dose of complement (about 1.2 $C'H_{50}$) and varying amounts of antigen and antiserum. In these experiments, dilution series of the reagents were prepared using grade A serological pipettes and the tests were performed in tubes. The antigens were purified 140S components of FMDV strains OV1 and O_1(Lom) and the antiserum was guinea pig serum produced against live guinea pig-adapted OV1 virus. The results are illustrated in Figures 1.1 to 1.5, the first four figures being homologous reactions with the OV1 strain.

(i) Variation of amount of antigen with constant antiserum

Figure 1.1 shows the effects of varying the amount of antigen in a series of mixtures, each with the amount of antiserum constant. For any curve representing constant antiserum, the response to increasing antigen is at first a linear increase in the amount of complement fixed, followed by a region of maximum fixation where the proportion of antigen to antibody is optimal and then a range of relative antigen excess with a progressive decrease in fixation. Smaller amounts of antibody gave a shallower slope in the zone of

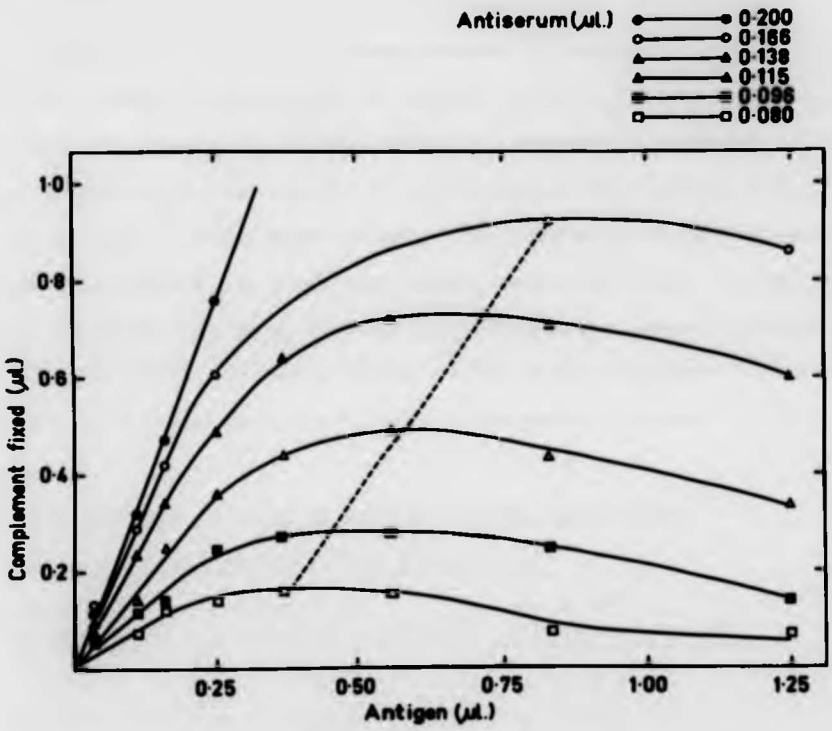


Figure 1.1. The effect of varying the amount of antigen on the amount of complement fixed. Each curve represents a constant amount of antiserum.

linear response and resulted in a lower maximum fixation.

The linear response is in the region of antiserum excess and it is apparent that under these circumstances the relationship of amount of antigen to the amount of complement fixed is directly proportional. In the region of maximum fixation the slope is small, so that the optimum amount of antigen for a particular amount of antiserum is poorly defined. However it is clear that the amount of antigen which is optimal, increases with an increase in the amount of antiserum in the mixture and with the amount of complement fixed. The broken line in Figure 1.1 suggests the possibility that the relationship is linear. The line was so placed to pass through points with an almost constant ratio of antigen to antiserum (Table 1.1), and it appears reasonable to assume that these points represent optimum amounts of antigen.

Table 1.1 Amounts of antigen and antiserum in optimum proportion mixtures, derived from Figure 1.1

Amount of antigen (µl)	Amount of antiserum (µl)	Ratio of antigen:antiserum
0.40	0.080	5.0
0.47	0.096	4.9
0.58	0.115	5.0
0.72	0.138	5.0
0.83	0.160	5.0

(ii) Variation of amount of antiserum with constant antigen

Figures 1.2 and 1.3 represent the effect of varying the amount of antiserum in a series of mixtures, each containing constant antigen. Figure 1.2 is derived from the same experiment

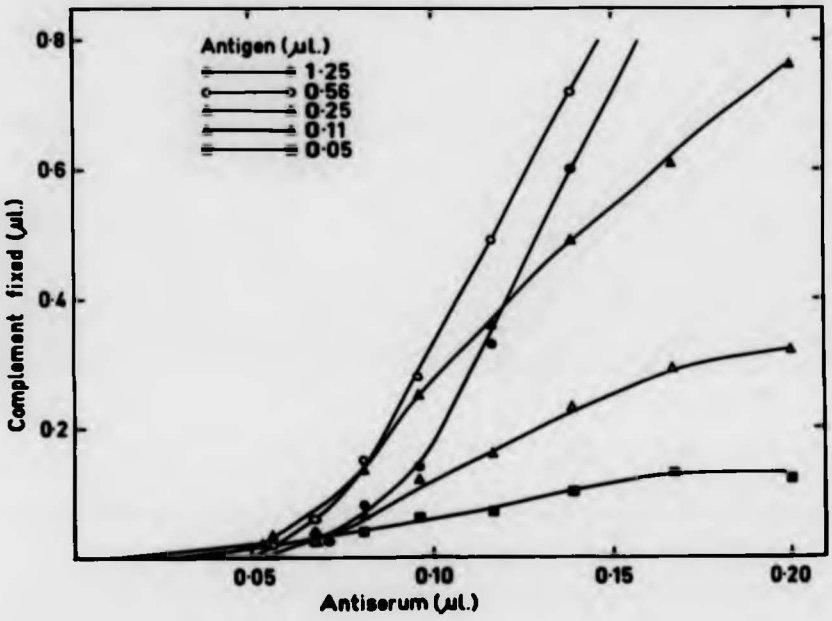


Figure 1.2. The effect of varying the amount of antiserum on the amount of complement fixed. Each curve represents a constant amount of antigen.

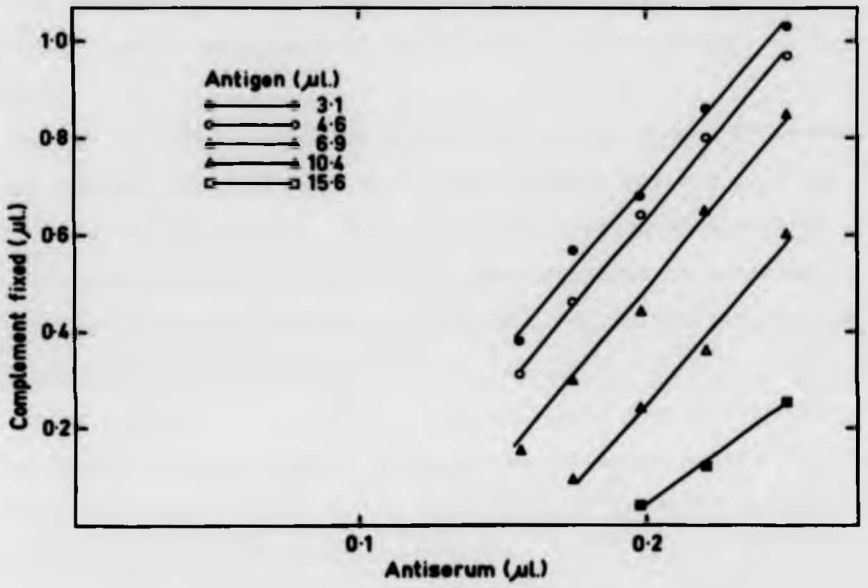


Figure 1.3. The relationship of the amount of antiserum to the amount of complement fixed. The lines represent constant antigen amounts over a range from close to optimal to a greater than fourfold antigen excess.

as Figure 1.1 and examination of Figure 1.1 will reveal that the lines of constant antigen amounts in Figure 1.2 of 0.25 µl., 0.11 µl. and 0.05 µl., represent antigen below the optimum over the range of amounts of antiserum used. While these curves are sigmoid, the lines representing 1.25 µl. and 0.56 µl. of antigen are linear over a large part of their range and are almost parallel.

Figure 1.3 from a separate experiment, shows lines of constant antigen amounts from 3.1 µl., which was close to optimal over the range of antiserum used, to 15.6 µl., which represents a greater than four-fold antigen excess. The linear response to increasing antiserum with constant antigen is apparent, as is the parallelism of all but one of the lines.

It is evident from Figures 1.2 and 1.3 that the relationship of complement fixed to amount of antiserum, although linear under certain conditions, is not one of direct proportionality since the lines do not pass through the origin.

(iii) Variation of amounts of antigen and antiserum while maintaining them in optimal proportions

Figure 1.4 is derived from Figure 1.1 by plotting the points of maximum fixation of complement for each level of antiserum. The graph indicates a linear response between the amount of complement fixed and the amount of antiserum in each optimum proportion mixture. It demonstrates that, at least within this range of fixation, linear interpolation or extrapolation from two or more points could be used to ascertain the amount of antiserum required to fix a certain defined amount of complement in the presence of an optimum amount of antigen. If the amount of

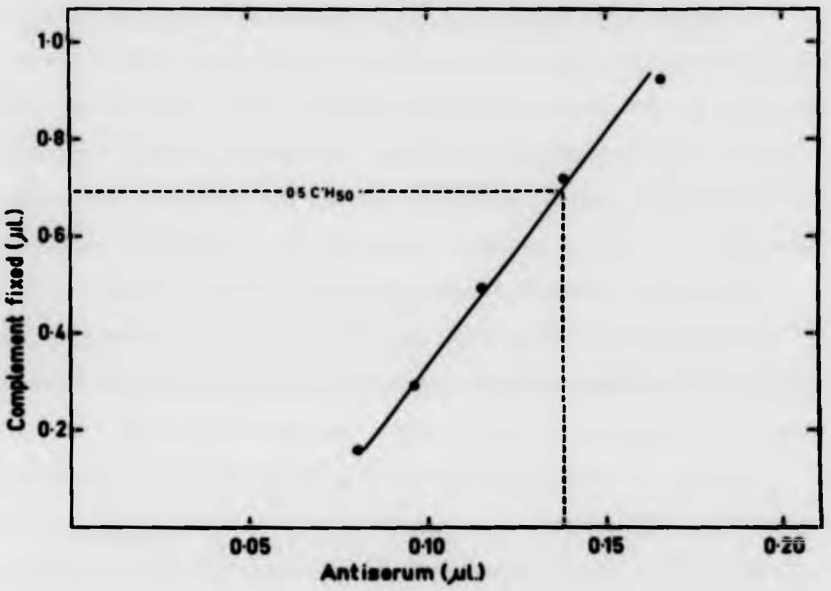


Figure 1.4. The relationship of the amount of antiserum to the amount of complement fixed in the presence of an optimal amount of antigen.

complement is taken as 0.5 $C'H_{50}$ (0.69 µl. in Figure 1.4), then 0.138 µl. of antiserum in a volume of 500 µl. is required to fix this amount of complement, i.e. the antiserum titre can be defined as 0.138/500 or 1/3623.

(iv) Reactions with homologous and heterologous antigens

Experiments carried out using a strain-heterologous antigen (O_1Lom. antigen with OV1 antiserum) demonstrated that a similar pattern of fixation occurred. However a greater amount of antiserum was required in the heterologous system, compared with the homologous system, for the same level of maximum complement fixation. Homologous and heterologous antigens in suitable ranges of serial two-fold dilutions were each tested against two levels of antiserum, pre-titrated to give maximum complement fixation in the desired range. The amount of complement fixed in each mixture was calculated and curves analogous to those of Figure 1.1 were drawn for both antigens at each level of antiserum. From these curves the amounts of complement fixed with optimum antigen were determined and plotted (Figure 1.5). The antiserum titres for the homologous and heterologous reactions were then found by linear interpolation as described above. Thus, the antiserum titre with homologous antigen (1/3677) was greater than with the heterologous antigen (1/1416).

1.3 (b) Differentiation of FMDV Strains in Tube and Microplate Tests

Using the principles of CF demonstrated in the system described above, comparative tests were carried out in tubes and in microplates, to evaluate the applicability of the method to FMDV strain differentiation.

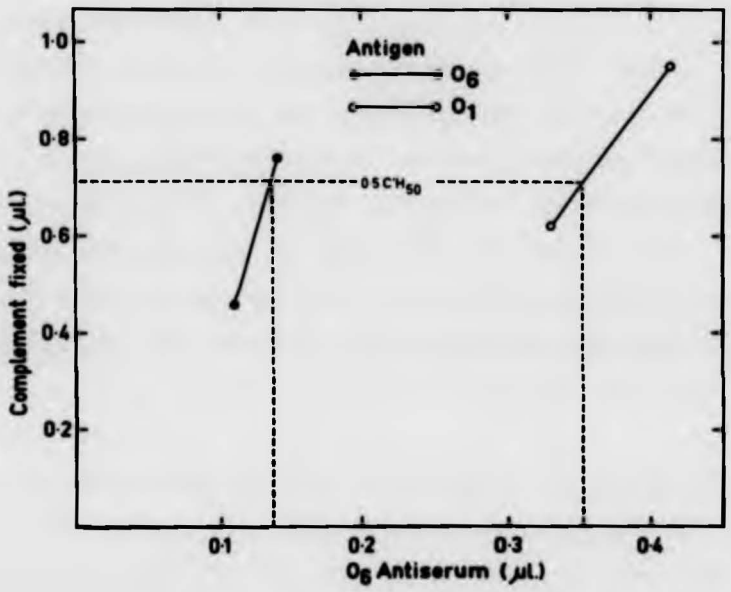


Figure 1.5. The graphical estimation of titres of O_6 (OV1) antiserum with homologous and heterologous (O_1 Lombardy) antigens.

To compare two strains in a tube test, each antigen was used in a series of two-fold dilutions over a pre-determined range which would demonstrate an optimum dilution giving maximum fixation. Antisera to each strain were tested at two dilutions against each antigen, with the amount of complement constant at approximately 1.2 $C'H_{50}$.

Suitable control mixtures were included in all tests, comprising complement alone and complement plus antigen or antiserum, at the dilutions used in the test. The amount of complement fixed with the optimum dilution of antigen against each dilution of antiserum was calculated and appropriate corrections made for pro- or anti- complementary activity in control mixtures. The titres of each antiserum with both antigens were then determined graphically as described above in Section 1.3 (a).

Comparative microplate tests were carried out using $5C'H_{50}$ as the constant complement dose, established by pre-titration in plates and equivalent in concentration to $5C'H_{50}$ in the tube test. Control mixtures were again included in all tests with complement at 5, 2.5 and 1.25 $C'H_{50}$ alone and with antigens and antisera at all dilutions used in the test.

To compare two strains in a microplate test, each antigen was used in a series of two-fold dilutions, as in the tube tests, and tested against each antiserum in a series of 1.5-fold or two-fold dilutions. The antiserum titre was determined as the dilution of serum which fixed 4 of the 5 $C'H_{50}$ with an optimum amount of antigen, i.e. the highest dilution of antiserum in which 50% of the sensitised erythrocytes remained unlysed. Where

necessary, interpolation between two wells containing more than 50% and less than 50% of unlysed cells was made by expressing the antiserum titre as the geometric mean of the two dilutions.

To obtain a quantitative estimate of the relationship between two strains, the method used was that described by Ubertini *et al.* (1964) and popularly applied since then to FMDV strain differentiation.

For two antigens, A and B, and their respective antisera, a and b, the following values are determined:-

$$r_1 \text{ (antiserum a)} = \frac{Ba}{Aa},$$

where Ba represents the reciprocal of the titre of antiserum a with the heterologous antigen and Aa represents the reciprocal of the homologous titre of the same antiserum;

similarly, r_2 (antiserum b) = $\frac{Ab}{Bb}$.

The antigenic relationship between the strains (R) is then determined by the formula

$$R = 100\sqrt{(r_1 \cdot r_2)}\ \%$$

(i) Comparative patterns in tube and microplate tests

In all of the tests carried out, the pattern of fixation in microplates was comparable to that in tubes, as illustrated by Table 1.2. An optimum dilution of antigen was always demonstrable in tube tests. However, in microplate tests the antigen optimum was often represented by a range of dilutions, probably owing to the semi-quantitative nature of the end-point determinations in microplates.

Table 1.2 Comparative complement-fixation tests in tubes and microplates

(a) Microplate tests - the numbers represent a visual score of the percentage of erythrocytes remaining unlysed: 4 = 100%. 3 = 75%, 2 = 50%, 1 = 25%.

Antigen (reciprocal of dilution)		Antiserum (reciprocal of dilution) A6003 16	32	64	128	256	512	A6900 16	32	64	128	256	512
A6003	32	4	4	4	4	-	-	4	4	4	-	-	-
	64	4	4	4	4	-	-	4	4	4	-	-	-
	128	4	4	4	4	2	-	4	4	4	2	-	-
	256	4	4	4	4	2	-	4	4	4	1	-	-
	512	2	2	2	2	-	-	-	-	-	-	-	-
A6900	32	4	4	4	-	-	-	4	4	4	4	-	-
	64	4	4	4	-	-	-	4	4	4	4	1	-
	128	4	4	4	-	-	-	4	4	4	4	1	-
	256	3	1	1	-	-	-	2	3	3	2	-	-
	512	-	-	-	-	-	-	-	-	-	-	-	-

(b) Tube tests - the results are shown as the percentage of lysis in each mixture

Antigen (reciprocal of dilution)		Antiserum (reciprocal of dilution) A6003 160	200	520	650	A6900 240	300	520	650
A6003	256			21	59	33	58		
	512			15	46	11	36		
	1024			27	55	18	36		
	2048			55	68	50	58		
	4096			70	76	71	73		
A6900	128	24	51					42	69
	256	22	47					20	50
	512	33	54					16	41
	1024	55	66					39	50
	2048	71	75					65	69

Where inhibition of fixation in antigen excess was not observed in microplate tests, the demonstration of an optimum amount of antigen was accepted on the basis of at least two antigen dilutions indicating the same maximum antiserum end-point.

The results shown in Table 1.2 also indicate that in both systems, the optimum dilution of an antigen was generally very similar for two different antisera, always being within one two-fold interval for both. This was a consistent finding in all tube and microplate tests discussed in this Section.

(ii) Reproducibility of results

Tables 1.3 and 1.4 summarise the results of microplate and tube tests respectively. The ranges and mean values of the serum titres and of $\underline{r}$ and $\underline{R}$ were obtained from the values for individual tests and, where microplate tests were performed with replicates, the values for $\underline{r}$ and $\underline{R}$ were derived from all combinations of the appropriate replicate titres within each test.

The range of variation in the results was lower in tube tests than in microplates. The maximum variation from a mean value for $\underline{R}$, as a percentage of the mean, was 7% in tube tests and 43% in microplate tests. The values for $\underline{R}$ in comparative tests were slightly greater, but not significantly so, in microplate tests than in tube tests.

It is apparent that the range of variation in an antiserum titre in tube tests was greater than the variation in a value for $\underline{R}$. It appeared that the main source of variation in anti-serum titres was a variation in the susceptibility of different

Table 1.3 Antiserum titres and values for r and R obtained in microplate tests

Parameters are shown as mean values with the range in brackets

(1) OV1 versus O_1Lom. (three tests, two in duplicate)

(2) A6003 versus A6900 (two tests, one in duplicate and one in triplicate

	Antigen	Antiserum	Antiserum titre	r	R
(1)	OV1	OV1	1072 (994-1220)	0.54 (0.44-0.66)	40% (35-47%)
	O_1Lom	OV1	575 (441-661)		
	O_1Lom	O_1Lom	562 (397-661)	0.30 (0.30)	
	OV1	O_1Lom	166 (118-196)		
(2)	A6003	A6003	272 (256-362)	0.36 (0.25-0.50)	42% (30-60%)
	A6900	A6003	97 (91-102)		
	A6900	A6900	288 (256-324)	0.50 (0.35-0.71)	
	A6003	A6900	145 (128-162)		

Table 1.4 Antiserum titres and values for r and R obtained in tube tests

Parameters are shown as mean values with the range in brackets

(1) OV1 versus O_1Lom. (4 tests)
(2) A6003 versus A6900 (3 tests)
(3) Asia 1 C_2 versus Asia 1 415 (3 tests)
(4) Asia 1 C_2 versus Asia 1 387 (2 tests)
(5) Asia 1 415 versus Asia 1 387 (2 tests)

	Antigen	Antiserum	Antiserum titre	r	R
(1)	OV1	OV1	3548 (2564-3846)	0.37 (0.34-0.42)	
	O_1Lom	OV1	1318 (1097-1429)		30% (28-32%)
	O_1Lom	O_1Lom	1862 (1724-1887)	0.26 (0.23-0.26)	
	OV1	O_1Lom	447 (427-485)		
(2)	A6003	A6003	589 (552-602)	0.28 (0.28-0.30)	
	A6900	A6003	166 (153-178)		37% (36-38%)
	A6900	A6900	562 (538-658)	0.49 (0.47-0.52)	
	A6003	A6900	295 (281-311)		
(3)	Asia1 C_2	Asia1 C_2	195 (180-213)	1.00 (0.97-1.04)	
	Asia 1 415	Asia1 C_2	195 (174-221)		83% (82-84%)
	Asia 1 415	Asia 1 415	145 (127-152)	0.69 (0.67-0.71)	
	Asia1 C_2	Asia 1 415	105 (94-122)		
(4)	Asia1 C_2	Asia1 C_2	195 (180-213)	0.95 (0.95)	
	Asia 1 387	Asia1 C_2	191 (177-202)		101% (100-101%)
	Asia 1 387	Asia 1 387	85 (79-100)	1.07 (1.06-1.08)	
	Asia1 C_2	Asia 1 387	95 (86-108)		

/Table continued overleaf

Table 1.4 Continued

	Antigen	Antiserum	Antiserum titre	r	R
(5)	Asia 1 415	Asia 1 415	145 (127-152)		
	Asia 1 387	Asia 1 415	87 (81-94)	0.64 (0.62-0.65)	80% (78-83%)
	Asia 1 387	Asia 1 387	85 (79-100)		
	Asia 1 415	Asia 1 387	89 (78-99)	1.00 (0.99-1.00)	

batches of erythrocytes causing the value of $1C'H_{50}$ to vary considerably between tests, only when different batches of erythrocytes were used. The values of r and R were affected much less by these errors, since they are proportional values.

In the microplate tests of OV1 v. O_1Lom., 1.5-fold antiserum dilutions were used. Since the range of variation of antiserum titres within tests was similar to those for tests of A6003 v. A6900, in which two-fold antiserum dilutions were used, it would appear that there is no advantage in using the closer dilution interval.

(iii) Antigenic relationships of the strains

From tests in tubes, the mean values for R between OV1 and O_1Lom. was 30% and between A6003 and A6900 was 37%. In both of these comparisons, antigenic differences were detectable with either antiserum but were quantitatively different, i.e. the values of r_1 and r_2 were unequal.

Two of the Asia 1 strains appeared to be identical (Asia 1 C2 v. Asia 1 387, R = 101%) and the third strain, Asia 1 415 was different but held a similar relationship to both of the other two (Asia 1 C2 v. Asia 1 415 R = 83%; Asia 1 387 v. Asia 1 415 R = 80%).

There was very little difference in the titres of either the Asia 1 C2 or the Asia 1 387 antisera when tested against any of the three strains, so that antigenic differences between the strains was detectable only with the Asia 1 415 antiserum. The two values for r obtained with this antiserum were of the same order (Asia 1 415 v. Asia 1 C2 r_1 = 0.69; Asia 1 415 v. Asia 1 387 r_1 = 0.64), which confirms the virtual identity of

the Asia 1 C2 and Asia 1 387 strains.

As the antisera to the Asia 1 strains were prepared using crude tissue culture antigens, it was possible that antibody to BHK cellular material could react with contaminating cellular debris. However, the method of antigen purification used should exclude host material and there was no fixation of complement by any of the three antisera, tested at the lowest dilutions used, against a similarly purified heterologous (type O) antigen.

1.4 DISCUSSION

The results in Section 1.3 (a) suggest a model for the fixation of complement in this system which is consistent with those obtained by other workers, using more precise methods in more closely defined systems. While the 140S component of FMDV is antigenically complex and the antiserum used in these experiments can react with a number of different antigenic sites (see Section 2), it would appear that complement fixation proceeds in a similar manner to that described by Osler *et al.* (1948) using a single antigen/antibody system (pneumococcal capsular polysaccharide). Shulman (1958) also provided experimental and theoretical evidence for a model of complement fixation by complexes of antibody, quinidine as a haptene and platelets, with strikingly similar characteristics.

However, the assumption made by Bradish *et al.* (1960 a), that in antigen excess there is a relationship of direct proportionality between the amount of antigen and the amount of complement fixed is contrary to these results. Since satisfactory experimental evidence for their assumption is

lacking it must be concluded that there is no valid basis for the CF test devised by these workers for FMDV strain differentiation.

The estimation of an antiserum titre, as described above in relation to Figure 1.4, is the basis of conventional chequerboard titrations. The response shown between the amount of antiserum and the amount of complement fixed is probably sigmoid (Wallace *et al*., 1950; de Almeida *et al*., 1952; Shulman, 1958) but the data in Figure 1.4 suggest that in this system it is reasonable to assume linearity over the range of fixation used. The use of only two points on this line (as in Figure 1.5) to determine an antiserum titre introduces the possibility of error in the estimate but was justified on the basis of technical simplicity. If the error in position of the points is small and large extrapolation is avoided, the error in the estimate of the antiserum titre should in practice be acceptable.

As shown in Figure 1.5, an antiserum will have a lower titre when reacting with a heterologous antigen than with its homologous antigen. It would appear that the expression of a difference in titres as a proportion (i.e. heterologous titre/homologous titre) is a valid and sensitive method of measuring the relationship between two antigens. This expression was the basis of the technique for differentiation of FMDV strains by Roumiantzeff *et al*. (1965 and 1966) in a system using $5C'H_{50}$ as the constant complement dose, and unpurified virus harvests for antigen. The use of a lower dose of complement as described for the tube tests in this Section enables a more accurate measurement of residual complement in antigen/antiserum mixtures

compared with that in antigen, antiserum and complement control mixtures.

The comparative results of tube and microplate tests illustrate the essential difference between the two methods. Microplate tests, while simple and rapid to perform and to replicate, are considerably less reproducible than tube tests which, however, require considerable care in the preparation and pre-titration of reagents. It would appear that the error in microplate tests would be quite acceptable for the routine differentiation of field strains of FMDV if the tests were sufficiently replicated. However the tube test provides a sensitive and accurate method for the detection of small antigenic differences.

Guerche et al. (1972) found that the two type A strains, A6003 and A6900, could not be differentiated by complement fixation. Since they found that the two strains were immunologically distinct, they concluded that complement fixation was an unsatisfactory method for detecting immunological variants. These authors used a complement fixation test in tubes in which the concentrations of antigen, antiserum and complement were all varied. Their results were not entirely consistent with the model of fixation described in this Section but this could be explained by their use of crude tissue culture harvests as antigens, since these will contain at least three complement fixing antigens, probably at different concentrations and with different strain specificities, as described in Section 2. The value for R determined in this Section (R = 37%) was of a similar order to that for the two type O strains (R = 30%) which are classified in the World Reference

Laboratory as different sub-types. It can only be concluded from this that the two strains, A6003 and A6900 are serologically distinct. The strains were used for further immunological studies, described in Section 3.

The differences between the type Asia 1 strain 415 and the two other strains, C2 and 387, were small but reproducible and in the light of the interrelationships between the three strains, would appear to be significant. It was demonstrated that the close similarities were not the result of fixation of complement by contaminating non-viral antigens. Nor is it likely that viral antigens other than 140S were reacting since other work (Section 2) suggests that such antigens - in particular, 12S sub-units resulting from degradation of the virion - are present in such preparations at only very low levels. These differences could, however, not be correlated with any of the other criteria by which the strains have been compared (Priston, 1972).

1.5 CONCLUSIONS

It has been shown that the fixation of complement by FMD virus and antibody in the system described follows the same principles as models developed in closely defined single antigen/antibody systems.

The concept of direct proportionality between the amount of complement fixed and the amount of antibody in antigen excess appears to be incorrect.

It was demonstrated that FMDV strains can be satisfactorily differentiated in CF tests, when the results are expressed as relationships derived from antiserum titres with a fixed amount of complement and optimum amounts of homologous and heterologous antigens.

The results obtained with tests carried out in microplates and in tubes are comparable, but in the latter case are more reproducible and provide a precise method for the identification of small antigenic differences.

SECTION 2. THE SEROLOGICAL ACTIVITY OF ANTIGENIC COMPONENTS OF FOOT-AND-MOUTH DISEASE VIRUS

2.1 INTRODUCTION

2.1 (a) The Structure of FMDV

The first measurement of the size of FMDV by Galloway and Elford (1931) gave an estimate of the size of the infective particle of 8 to 12 nm. The same authors later re-estimated the size as about 20 nm. (Elford and Galloway, 1937).

Traub and Pyl (1943) and Möhlmann (1943) demonstrated that centrifugation under conditions that sedimented 99% of the infectivity of a virus harvest, removed only 50 to 75% of the complement-fixing activity from the supernatant. Similar findings by Bradish et al. (1952), led them to postulate the existence of two complement-fixing components, the larger one (estimated as 20 nm.) being associated with all or most of the infectivity and the smaller component (estimated to be 7 nm.) possessing no detectable infectivity. Bradish et al. (1960 b) re-estimated the size of the infective component as about 25 nm. The two components shall be referred to by their sedimentation coefficients, the most reliable estimates for these being 140S for the infective particle (Trautman et al., 1959) and 12S for the smaller component (Talbot and Brown, 1972).

Bradish and Brooksby (1960) demonstrated that thermal degradation of the 140S component produced a smaller unit which behaved similarly in CF tests to the naturally occurring 12S component and Brown and Cartwright (1961) produced a similar component by mild (pH 6.5) acid disruption of the virion.

In 1968, Vande Woude and Bachrach found that disrupted virus separated into multiple bands on polyacrylamide gel

electrophoresis. Wild et al. (1969) provided evidence that such bands represented different polypeptides. Burroughs et al. (1971) demonstrated the separate identity of four polypeptides with molecular weights of 34, 30, 26 and 13.5 x 10^3 in sodium dodecyl sulphate polyacrylamide gels. They also showed that the 12S component produced by mild acid disruption of the virion was deficient in one polypeptide which was identified as the major component of a separate aggregate.

The virion structure of several picornaviruses has been studied and a comparative review has been made by Rueckert (1971). Talbot and Brown, (1972) proposed a model for FMDV demonstrating a close similarity in the structure of this virus with other members of the family. They showed that the four polypeptides (VP1 to VP4) were present in the ratio of 1:1:1:0.5. In this model the virion is comprised of twenty 12S sub-units and thirty molecules of VP4. Each 12S sub-unit contains three each of VP1, VP2 and VP3 and represents one of the triangular faces of an icosahedron. The VP4 molecules are situated singly on the lines of apposition of the faces of the icosahedron. Within this protein capsid is the viral RNA, comprising 31% of the weight of the virus particle (Bachrach et al., 1964).

2.1 (b) The Antigenic Properties of FMD Viral Components

There is ample and convincing evidence that the 140S virus particle is the most effective antigenic component for the stimulation of neutralising antibody production (Randrup, 1954; Wild and Brown, 1968). Virus rendered non-infective is immunogenic provided the capsid remains intact, and the potency of inactivated vaccines derived from some unstable strains can be enhanced by stabilisation of the 140S particle by treatment

with formaldehyde (Rowlands et al., 1972; Mowat et al., 1973).

The antigenic activity of the 140S component is apparently type specific. Brown and Crick (1958) found that 140S particles gave precipitin lines in agar gel diffusion tests only against homotypic antiserum and that the reaction could not be prevented by mixing the virus with heterotypic antiserum. Bradish and Brooksby (1960) showed that the 140S component reacts only with homotypic antiserum in complement fixation tests.

However, serological cross-reactions between 140S particles of different sub-types are readily demonstrable. They were demonstrated in CF tests with unfractionated virus harvests by Traub and Mőhlmann (1946) and Brooksby et al. (1948 a) and were identified as a property of the 140S particle by Bradish and Brooksby (1960).

Booth and Pay (1973) demonstrated that virus harvests of FMDV type SAT 2 under certain conditions haemagglutinate guinea pig erythrocytes. Purified 140S preparations of different sub-type strains possessed haemagglutinating activity and antigenic differences were demonstrable in haemagglutination-inhibition tests.

Examination of the 12S component, separated from virus harvests (natural 12S) or obtained by disruption of the virion (artificial 12S) revealed that it had antigenic properties related to, but different from those of the 140S component. It has a negligible ability to stimulate neutralising antibody production (Brown 1973) and absorption of IgG antibody with 12S component does not reduce its neutralising activity (Rowlands et al., 1971).

Cowan (1968) found that guinea pigs immunised with either

140S or 12S components produced antibodies reactive with both components. However, Cartwright (1962) and Cowan (1968) demonstrated that absorption of antisera having such dual reactivity, with an excess of 12S component would reduce but not eliminate the anti-140S activity, indicating a partial antigenic identity.

In contrast to the purely homotypic activity of the 140S particle, cross-reactions between types are demonstrable with the 12S component. Brown and Crick (1958) found that lines of precipitation produced in agar gel diffusion tests with 12S antigen and homotypic antiserum, could be prevented by mixing the antigen with heterotypic antiserum. However, diffusion of 12S antigen against heterotypic antiserum did not produce a visible precipitate. Bradish and Brooksby (1960) showed that the type-specific 140S antigen developed heterotypic activity in CF tests after thermal degradation.

Bradish and Brooksby (1960) also examined the sub-type specificity of their separated U-fractions (assumed to be 12S component) and D-fractions (140S component). They concluded from their observations that the 12S component has less sub-type specificity than the 140S component.

Non-infective, empty capsids which sediment at 75S were identified by Graves et al. (1968). They differed from 140S particles in being resistant to acid degradation to the 12S component, and in reacting with anti-12S serum which possessed no anti-140S activity. Their presence is quantitatively significant in some virus strains (up to 85% of the total complement-fixing activity in harvests of one of the strains studied by Graves et al., 1968), and they stimulate as much

neutralising antibody as 140S particles provided they have first been fixed with formaldehyde (Rowlands, D.J., Sangar, D.V. and Brown, F.; quoted by Brown, 1973).

Wild and Brown (1967) demonstrated that the treatment of FMDV with trypsin considerably reduced its infectivity and its ability to stimulate neutralising antibody production, without altering its gross morphology. Wild et al. (1969) showed by agar gel diffusion tests that trypsin-treated virus was less reactive than untreated virus with homologous antiserum. They also demonstrated that virus treated with the enzyme was no longer able to attach to susceptible cells. Burroughs et al. (1971) and Strohmaier and Adam (1974) showed that trypsin treatment affected only one polypeptide (VP1). Rowlands et al. (1971) found that trypsin-treated virus only stimulated 1% to 10% of the level of neutralising antibody produced by untreated virus. They concluded that two distinct types of neutralising antibody are produced by complete particles and that the major one is not stimulated by trypsin-treated virus.

Booth and Pay (1973) showed that the haemagglutinating activity of type SAT 2 viruses was common to both 140S and 75S particles but was absent from the 12S component and from trypsin-treated 140S particles.

Brown and Smale (1970) studied the reactions of FMDV with IgG and IgM by agar gel diffusion tests and by electron-microscopy. They suggested that the surface of the virion possesses three types of combining site. One type of site appeared to be on the faces of the particle and was common to the intact virion, the trypsin-treated virus and the 12S component. The two other types of site were situated at regular intervals,

probably at the icosahedral vertices, but only one was present on the trypsin-treated virion and by implication, neither of them on the 12S sub-unit. Cowan (1969) demonstrated that IgM could detect differences between strains in agar gel diffusion tests which were not detectable using IgG. This greater differentiating ability of IgM was also demonstrated by Brown and Smale (1970) since they found that IgM reacted only with the antigenic site absent from the 12S and trypsin-treated particles.

In 1973, Talbot et al. demonstrated that the polypeptide VP4 from strains of all seven types of FMDV migrated to a similar position in polyacrylamide gel electrophoresis preparations, although the polypeptide separation patterns were otherwise different and characteristic of the virus type. They also showed that VP4 reacts with heterotypic as well as homotypic antisera in complement-fixation tests.

Cowan and Graves (1966) identified an antigen in infected tissues and tissue culture harvests which, although specific for FMD infection, appeared to not be a structural component of the virion. It is possibly an enzyme necessary for viral RNA replication and is termed virus infection-associated (VIA) antigen. It was found that VIA antigen of type A cross-reacted in CF tests with antisera of all types except SAT 2.

In this Section, experiments are described in which preparations of viral antigens were tested by CF. The various antigenic components were compared to determine their relative potency, their inter-type and intra-type serological specificity, and the effect on their activity of long and short incubation periods in the CF test.

2.2 MATERIALS AND METHODS

2.2 (a) Antigens

Antigens were prepared from the following strains:

type O : OV1, O_1 Lombardy;

type A : A6003, A6900;

type SAT 1: RV 11/37;

type SAT 3: RV 7/34.

140S antigens were prepared from virus harvests by concentration, pelleting and sucrose density gradient centrifugation, as described in Appendix 2 (b).

75S antigens (RNA-free capsids) were prepared as described in Appendix 2 (c), with the final purification again carried out by sucrose density gradient centrifugation.

Preparations of trypsin-treated virus were made by treating the re-suspended pellet material with trypsin prior to the final purification (Appendix 2 (d)).

Artificial 12S antigen was prepared by degrading purified virus followed by a further centrifugation through sucrose density gradients (Appendix 2 (e)).

The separation of natural 12S antigen and VIA antigen from the supernatant remaining after pelleting the 140S antigen, was achieved by a modification of the method described by Cowan and Graves (1966) using diethylaminoethyl (DEAE)- cellulose chromatography. Sucrose density gradient centrifugation was then used for further purification (Appendix 2 (f)).

Some of the antigen preparations were labelled with radioisotopes as indicated in the text. The methods used are described in Appendix 2 (g).

2.2 (b) Antisera

Live-virus antisera were prepared in guinea pigs by the method described by Brooksby (1952) (Appendix 3 (a)).

Antisera specific for 140S antigen were prepared by adsorbing live-virus antiserum with acid-degraded purified virus. The method followed was that of Brown and Smale (1970) and is described in Appendix 3 (b).

Anti-140S sera were prepared against purified, inactivated 140S antigens by the method described in Appendix 3 (c).

An antiserum was prepared against artificial 12S antigen as described in Appendix 3 (d).

2.2 (c) Complement-fixation Tests

CF tests for the assay of sucrose density gradient fractions and DEAE-cellulose column elution fractions were performed in microplates by standard methods as described in Appendix 4.

To evaluate the serological specificity of the antigen preparations, CF tests were carried out in tubes using the materials and methods described in Section 1.2. Long-fixation CF tests were also carried out, in which primary fixation was allowed to proceed for 18 hours at 4°C. All other conditions were identical to those for the short-fixation (30 minutes at 37°C) tests.

CF tests to determine the total complement-fixing activity (potency) of antigens were carried out in tubes, using the same protocol as for the specificity tests, with either long or short fixation periods. Antigens were tested in two-fold dilution series, against a fixed amount of antiserum at a concentration of at least four times the antiserum titre (as

defined in Section 1.3) and a constant amount of complement of approximately 1.2 $C'H_{50}$. Under these conditions the amount of complement fixed is directly proportional to the amount of antigen present (Section 1). The antigenic potency was measured in complement-fixing units (cfu), one cfu. being the amount of antigen in 500 µl. which would fix 0.5 $C'H_{50}$ of complement.

2.3 RESULTS

2.3 (a) Complement-fixation Tests with 140S Antigens

Tests for type and sub-type specificity were carried out with 140S antigens purified on sucrose density gradients. Figure 2.1 shows a typical gradient profile of complement-fixing activity and UV-absorbance at 259 nm. It is evident that the virus peak is well separated from the slower sedimenting cellular contaminants and that the CF test routinely used for the assay of sucrose density gradients is a satisfactory method of identifying the virus peak.

Type O (OV1) and type A (A6003) 140S antigens were tested for activity against heterotypic antisera (types A and O respectively) in CF tests using a fixation period of 18 hours at 4°C. The antisera were used at 100 times the concentration of their homologous 140S titres. No fixation was detected with either of the antigens at the dilutions giving optimum fixation with their homologous antisera. However, at higher concentrations, a variable amount of fixation was detected, depending on the age of the antigen. A preparation of 140S antigen of the strain A6003 tested on the day of purification showed no fixation with OV1 antiserum except at a concentration 64 times greater than the homologous antigen titre. A second 140S antigen of the same strain, tested 7 days after preparation,

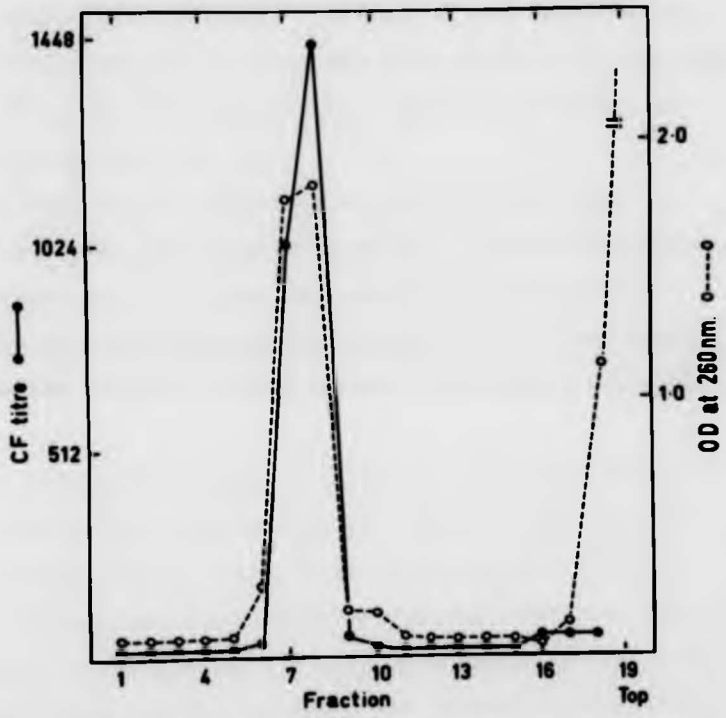


Figure 2.1. CF activity and UV absorbance of fractions from a sucrose density gradient for purification of 140S antigen, strain A6003.

fixed complement with OV1 antiserum with an antigen titre only 16 times lower than the homologous antigen titre. Because of the complete lack of cross-reaction with antigens at their optimum dilutions for homologous reactions and the very high concentrations of freshly-prepared 140S antigen required to demonstrate any heterologous fixation, it was concluded that such fixation was due to antigenic sites exposed on degradation of the virion with time; i.e. that intact virions have no heterotypic CF activity.

The results of a number of CF tests for sub-type specificity were presented in Section 1. Further experiments were carried out to compare the sub-type specificity of reactions in short-fixation (30 minutes at 37°C) and long-fixation (18 hours at 4°C) CF tests. The results are shown in Table 2.1

It is clear from these results that the specificity of the 140S reactions was lower in the long-fixation than in the short-fixation tests. This could be the result of a more complete cross-reaction between antigen and specific anti-140S antibody. Alternatively, it could be a manifestation of an increased cross-reaction between 140S antigen, either intact or partially degraded to 12S antigen, and antibody reactive to both 140S and 12S antigens. To clarify this, long fixation cross-CF tests were carried out between OV1 and O_1 Lombardy strains, using antisera which had been adsorbed with acid-degraded virus and which possessed no detectable anti-12S activity.

Table 2.1 Comparative short-fixation and long-fixation CF tests for sub-type specificity of 140S antigens

CF test	Antigen	Antiserum	Antiserum titre	r	R
Short-fixation	OV1	OV1	3659	0.35	30%
	O_1Lom	OV1	1271		
	O_1Lom	O_1Lom	1786	0.25	
	OV1	O_1Lom	450		
Long-fixation	OV1	OV1	13846	0.49	41%
	O_1Lom	OV1	6729		
	O_1Lom	O_1Lom	6207	0.35	
	OV1	O_1Lom	2167		
Short-fixation	A6003	A6003	596	0.29	37%
	A6900	A6003	171		
	A6900	A6900	670	0.46	
	A6003	A6900	308		
Long-fixation	A6003	A6003	3191	0.41	49%
	A6900	A6003	1320		
	A6900	A6900	3168	0.58	
	A6003	A6900	1852		

Table 2.2 Comparative cross-CF tests for sub-type specificity of 140S antigens using non-adsorbed and 12S-adsorbed antisera

Type of antiserum	$\underline{r}_1$ (OV1)	$\underline{r}_2$ (O_1Lom)	$\underline{R}$
Non-adsorbed	0.50	0.36	43%
12S-adsorbed	0.48	0.35	41%

The results in Table 2.2 indicate that the reactions with 12S-adsorbed antisera have virtually the same specificity as those with non-adsorbed antisera. Consequently, it would appear that the decreased specificity in the long-fixation tests is the result of a more complete cross-reaction of the 140S antigen with 140S-specific antibodies.

The 140S antigens of the OV1 and O_1 Lombardy strains were also compared in long fixation CF tests using anti-140S sera. The results were as follows:

$\underline{r}_1$ (OV1) = 0.37

$\underline{r}_2$ (O_1Lom) = 0.37

$\underline{R}$ = 37%

Although the value for $\underline{r}_1$ was significantly lower, the overall relationship, $\underline{R}$, was similar to the values determined with the live-virus antisera.

The results shown in Table 2.1 indicate that antiserum titres to 140S antigen were considerably greater in long-fixation than in short-fixation tests. As a mean of the four homologous reactions shown in this Table, the antiserum titre in a long-fixation test was greater by a factor of 4.5 than the corresponding titre in a short-fixation test.

A sample of purified 140S antigen was tested for antigenic potency in long and short fixation tests. The values obtained

were:

Long-fixation 7194 cfu.

Short-fixation 1613 cfu.

Therefore the potency determined by a long-fixation test was 4.5 times greater than that determined with short-fixation.

2.3 (b) The Sub-type Specificity of Empty Capsids

Empty capsids (75S antigen) of the strain A6003 were compared with 140S antigen of the same strain for sub-type specificity and for antigenic potency.

Three preparations of 75S antigen were made and compared in three separate experiments with fresh preparations of 140S antigen. In one experiment, the virus was grown in the presence of ^{14}C-labelled amino-acids and ^{3}H-labelled uridine to determine the relative antigenic potencies of the two particles, and to ascertain the maximum degree of contamination of the 75S antigen with 140S antigen (the reverse was not a problem, since the SDS used in the 140S purification procedure destroys 75S particles; D.J. Rowlands, personal communication). Figure 2.2 shows sucrose density gradient profiles of this preparation in terms of the ^{14}C and ^{3}H activity of the fractions.

Since the ^{14}C-labelled amino-acids are incorporated into the viral capsid and the ^{3}H-labelled uridine into the viral RNA, the lower peak in Figure 2.2 represents intact virus and the upper peak, empty capsids. This was confirmed by calculation of the sedimentation rate of the higher peak. Assuming a linear gradient, and a value of 140S for the lower peak, a value of 75S is obtained for the higher peak. The complement-fixing profiles of this gradient and of the other two preparative gradients for 75S antigen were essentially the same as the ^{14}C-activity profile

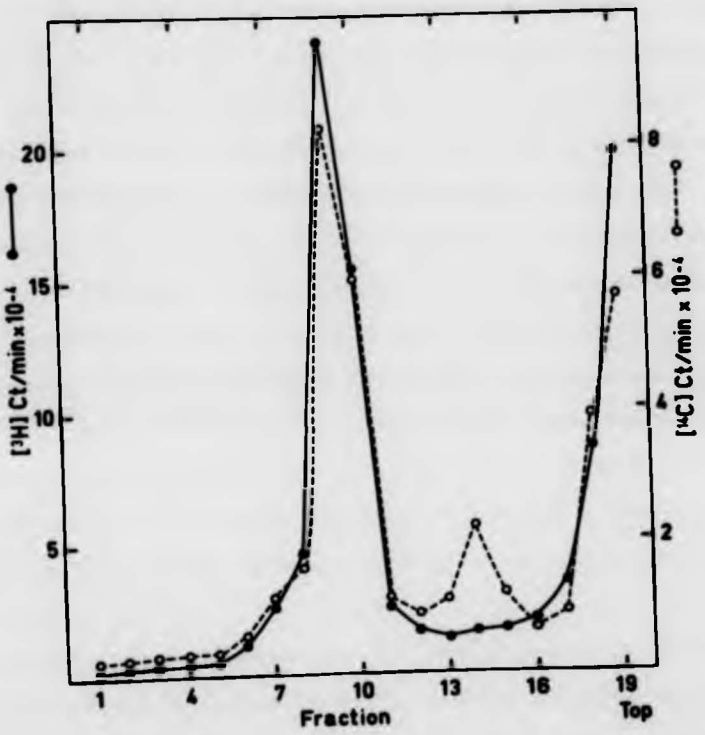


Figure 2.2. ^{3}H and ^{14}C activity of fractions from a sucrose density gradient for the preparation of 75S antigen, strain A6003.

of Figure 2.2.

The ratios of ^{3}H:^{14}C activity in the 140S and 75S peaks of the double-labelled preparation were 2.91 and 0.67 respectively. Thus the ratio of ^{3}H:^{14}C activity in the 75S peak is only 23% of that for the 140S peak. If these activities were due only to radio-isotopes incorporated into virus particles, the value of 23% would represent the amount of contamination of the 75S antigen with 140S particles. However, the profile of activity throughout the gradient would suggest that the majority of the ^{3}H activity in the 75S region was the result of unincorporated label. Consequently the degree of contamination was probably much less than 23%. Infectivity titrations by plaque assay (Appendix 6) gave values for the 140S and 75S peaks of $10^{9.2}$ and $10^{7.3}$ pfu./ml. respectively. The ratios of infectivity: ^{14}C activity in the two peaks were 1.9×10^{6} for the 140S and 9.1×10^{4} for the 75S peak. This indicates that the degree of 140S contamination of the 75S peak was 9.1/190, or approximately 5%.

In each of three experiments, 75S antigen and 140S antigen were tested by short-fixation CF tests in tubes, against the homologous antiserum, A6003 and a sub-type heterologous antiserum, A6900, to compare their sub-type specificity. The results are shown in Table 2.3. It can be seen that the titres of each antiserum with either 140S antigen or 75S antigen was virtually identical; i.e. the two antigens were equally reactive and had the same sub-type specificity.

Table 2.3 Homologous and heterologous antiserum titres with FMDV strain A6003 140S and 75S antigens

Antigen	Antiserum	Antiserum titre		
		Expt. 1	Expt. 2	Expt. 3
140S	A6003	546	524	543
75S	A6003	542	524	538
140S	A6900	256	288	271
75S	A6900	243	283	262

To determine the relative complement-fixing potency of the 140S and 75S antigens, the radio-actively labelled preparations were assayed for total CF activity. It is evident from Table 2.4 that the ratio of ^{14}C activity was virtually identical to that of the CF activity of the two antigens. This suggests that 140S and 75S particles have the same capability for fixing complement.

Table 2.4 ^{14}C activity and CF activity of FMDV strain A6003, 140S and 75S antigens

	^{14}C activity (ct./min.)	CF activity (cfu.)
140S antigen	8294	7.87
75S antigen	2213	2.08
Ratio	3.75	3.78

In the light of the virtual identity of the CF reactions with 140S and 75S antigens, it can be assumed that a small amount of contamination of the 75S antigen with 140S particles would not have affected the results. In the sub-type specificity tests, the fact that the A6900 antiserum titres were very slightly lower with the 75S antigen may be significant. Empty capsids are

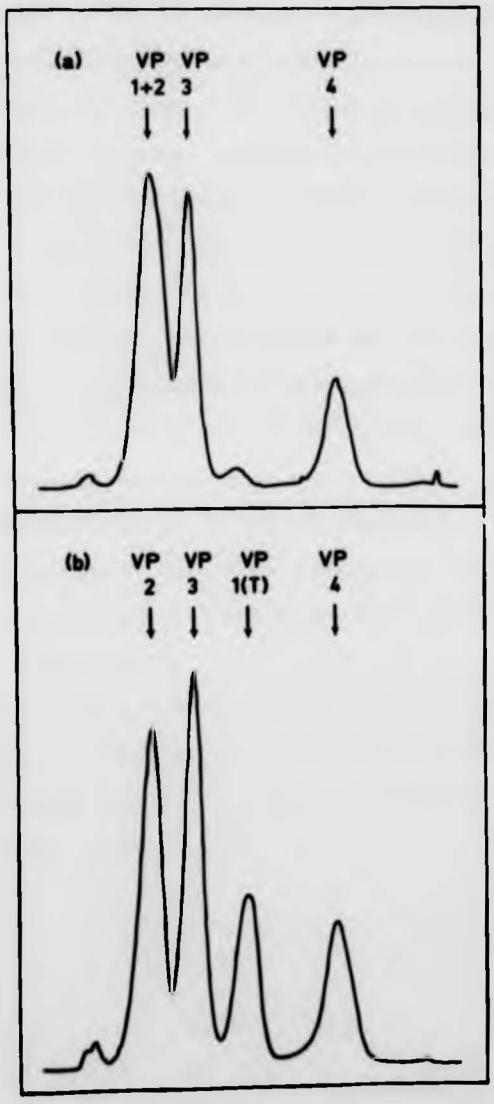
less stable than intact virions (D.J. Rowlands, personal communication), and a small degree of breakdown could alter the antigenic character of the particles, causing an altered reactivity with this antiserum.

2.3 (c) The Sub-type Specificity of Trypsin-treated Virus

Purified trypsin-treated 140S antigens (T-140S) were compared with normal 140S antigens of the same strain, for sub-type specificity. Three preparations of OV1 antigen and one of O_1 Lombardy were made and for each preparation the effectiveness of the trypsin-treatment was verified by demonstrating a decrease in infectivity of at least $10^{3.0}$ pfu./ml. of the treated part of a divided sample, compared with the untreated part.

One preparation of the OV1 strain was labelled with ^{35}S-methionine and experiments were carried out to confirm more fully the quantitative conversion of the virus to trypsin-treated particles. Samples of purified 140S and T-140S antigens were dissociated under denaturing conditions and the polypeptides were separated by polyacrylamide gel electrophoresis (Appendix 5). Preparation and electrophoresis of the gels was kindly carried out by Dr. H.G. Pereira. The gels were analysed by autoradiography and the developed autoradiographs scanned with a microdensitometer (Joyce, Loebl & Co. Ltd., Gateshead). The profiles obtained are shown in Figure 2.3 and they demonstrate that on trypsin-treatment a new polypeptide is obtained (VP1T) and that there is a decreased density of the peak consisting of VP1 and VP2. This is consistent with the evidence of Wild *et al.* (1969) and Burroughs *et al.* (1971) that treatment with the enzyme affects only VP1.

Figure 2.3. Polyacrylamide gel electrophoresis of strain OV1, (a) virus and (b) trypsin-treated virus. Migration from left to right.



Further evidence for the completeness of conversion to T-140S was obtained in an electrophoretic mobility experiment carried out with the assistance of Dr. R.A.J. Priston. The method is described in Appendix 7 and separation of proteins is obtained on the basis of their charge. Electrophoresis columns were run concurrently with 140S and T-140S samples, fractionated and analysed for radio-activity. The profiles shown in Figure 2.4 are characteristic of those obtained with normal and trypsin-treated particles of the OV1 strain (Priston, 1972). The lack of any activity in the T-140S profile in the region of the peak in the 140S profile, confirms that the virus had been quantitatively converted to T-140S. The divided nature of the T-140S peak is probably due to breakdown of the antigen to 12S subunits, since trypsin-treated virus is relatively unstable (D.J. Rowlands, personal communication). However, it is unlikely that such degradation occurred in the CF tests to any large extent since, unlike the CF test, the conditions of the electrophoretic mobility experiment (18 hours at 25°C) would favour deterioration of the antigen.

Short-fixation CF tests were carried out with 140S and T-140S antigens against homologous and sub-type heterologous (O_1Lom for OV1 antigens and OV1 for O_1Lom antigens) antisera. The results are shown in Table 2.5.

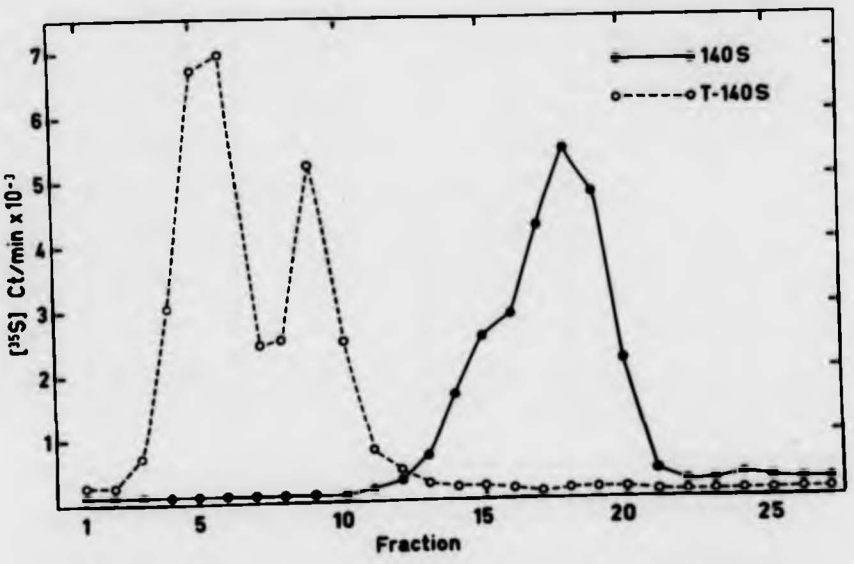


Figure 2.4. Comparison of electrophoretic mobilities of strain OV1 virus (140S) and trypsin-treated virus (T-140S). Migration from right to left.

Table 2.5 Homologous and heterologous antiserum titres obtained with 140S and T-140S antigens

Strain	Antigen	Antiserum	Antiserum titre		
			Expt. 1	Expt. 2	Expt. 3
OV1	140S	OV1	3000	2632	2941
OV1	T-140S	OV1	2182	1983	2308
OV1	140S	O_1Lom	523	402	459
OV1	T-140S	O_1Lom	523	398	441
O_1Lom	140S	O_1Lom	1659		
O_1Lom	T-140S	O_1Lom	1364		
O_1Lom	140S	OV1	923		
O_1Lom	T-140S	OV1	882		

From these results it can be seen that, whereas there was a significant decrease in the homologous antiserum titres (approximately 20%) with T-140S antigens, the heterologous antiserum titres were virtually the same with 140S and T-140S antigens; i.e. the trypsin-treated antigen had a decreased sub-type specificity. The slightly lower heterologous antiserum titres with T-140S antigen could be explained by a small degree of degradation of the antigen as referred to above in relation to 75S antigen.

It would appear that the antigenic site on VP1 which is affected by trypsin has a high degree of sub-type specificity. However, it is notable that the titres of both antisera were much greater with homologous T-140S antigen than with the heterologous antigens i.e. that the T-140S antigen still possessed considerable sub-type specificity. This would suggest that the

trypsin-sensitive antigenic site is not the only one which is sub-type specific.

2.3 (d) The Type and Sub-type Specificity of Natural and Artificial 12S Antigens

The final separation of natural 12S and VIA antigens was carried out by sucrose density gradient centrifugation. Figure 2.5 shows the profiles of complement-fixing activity of gradients used for the isolation of natural 12S antigen (N12S) from the initial eluate of a DEAE-cellulose column (Sample A) and the isolation of VIA antigen from the fractions eluted from the column with a NaCl gradient (Sample B). The major peak from Sample A was used as N12S antigen and the peak from sample B as VIA antigen. The identity of these antigens was confirmed on every occasion by heating a sample at 50°C for 30 minutes, which destroys VIA antigen (Cowan and Graves, 1966) but not 12S antigen. It is evident from Figure 2.5 that there was a certain amount of VIA antigen in sample A. However, separation of the two peaks was clear and so the N12S and VIA antigens were considered to be effectively separated from each other.

Artificial 12S antigen (A12S), prepared by acid degradation of the virion and purified on sucrose density gradients, sedimented at the same rate as N12S antigen. Figure 2.6 shows the result of co-sedimenting a trace amount of ^{14}C-labelled A12S antigen, with N12S antigen detected by CF activity.

Natural 12S antigens of the strains OV1 (type O), A6003 (type A), RV 11/37 (type SAT 1) and RV 7/34 (type SAT 3) were examined for type specificity in long-fixation CF tests. The results are shown in Table 2.6.

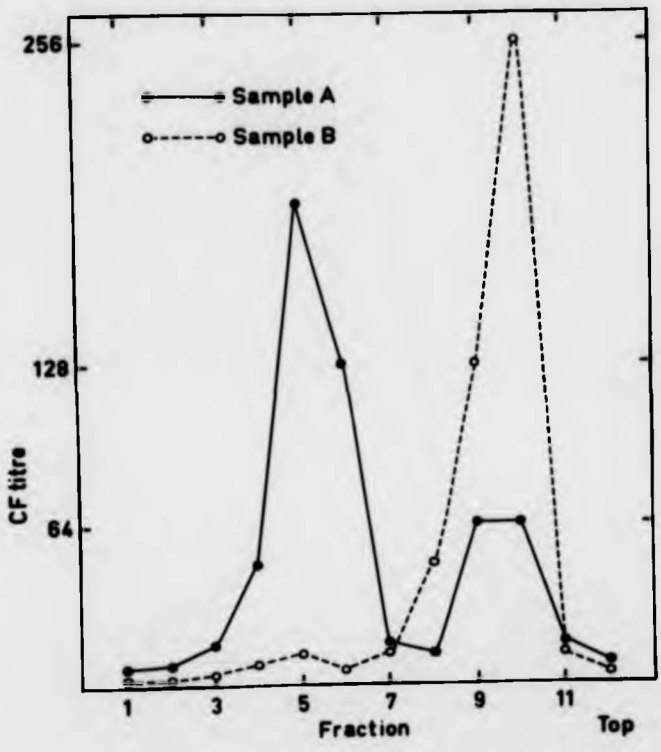


Figure 2.5. Comparison of the CF activities of fractions from sucrose density gradients for the preparation of natural 12S antigen from Sample A and VIA antigen from Sample B.

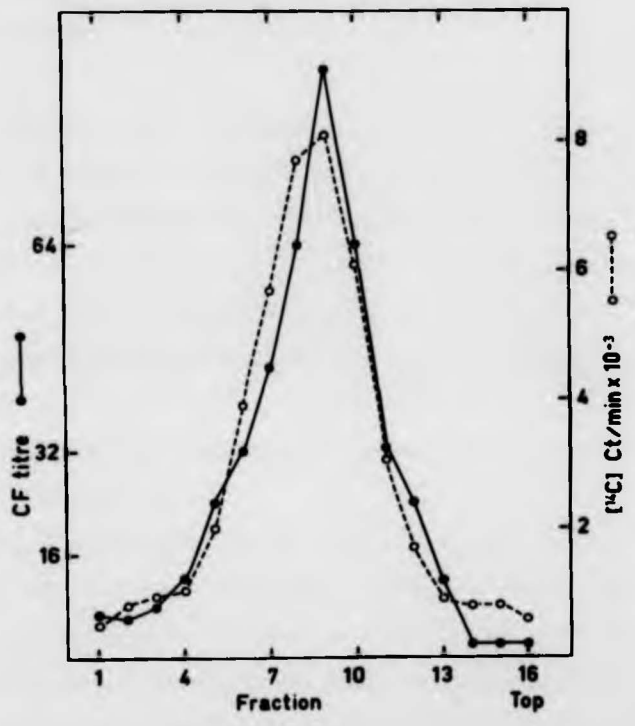


Figure 2.6. Co-sedimentation on a sucrose density gradient of strain OV1 natural 12S (CF activity) and artificial 12S (^{14}C activity) antigens.

Table 2.6 Relationships between the natural 12S antigens of types O, A, SAT 1 and SAT 3

Strains	r_1	r_2	R
OV1, A6003	0.12	0.09	10%
OV1, RV 11/37	0.02	0.02	2%
OV1, RV 7/34	0.09	0.07	8%

It is evident that heterotypic reactions occurred, but that they were very much lower than the homotypic reactions; i.e. the antigens possessed a very considerable degree of type specificity. The relationship between two strains of different European types (OV1 and A6003) was much closer than that between the type O and type SAT 1 strains (OV1 and RV 11/37), while that between type O and type SAT 3 strains (OV1 and RV 7/34) was intermediate, and not significantly different to that between the European strains.

Natural 12S antigens of the type O strains, OV1 and O_1 Lombardy and the type A strains, A6003 and A6900, were compared for sub-type specificity. The results of CF tests using long and short fixation periods are shown in Table 2.7.

Table 2.7 Sub-type relationships between natural 12S antigens of types O and A

Strains	Fixation period	r_1	r_2	R
OV1, O_1Lom	short	0.57	0.59	58%
OV1, O_1Lom	long	0.58	0.62	60%
A6003, A6900	short	0.46	0.46	46%
A6003, A6900	long	0.53	0.49	51%

It is evident that the natural 12S antigens possessed some sub-type specificity. The relationships shown in long-fixation tests were almost identical to those in short-fixation tests indicating virtually no decrease in specificity of the reaction when the long fixation period is employed.

Antiserum titres were considerably increased in long-fixation, compared with short-fixation tests. In the homologous reactions of the tests summarised in Table 2.7, the antiserum titres were an average of 5.4 times greater with long fixation.

The homologous antiserum titres with natural 12S antigens were considerably lower than with 140S antigens in both long and short fixation tests. Table 2.8 shows that the N12S antigen titres were generally three- to four-fold lower than the 140S titres.

Table 2.8 Homologous antiserum titres to 140S and N12S antigens

Strain	Antigen	Antiserum titre	
		Short-fixation	Long-fixation
OV1	140S	3659	13846
OV1	N12S	1163	5435
O_1Lom	140S	1786	6207
O_1Lom	N12S	588	2841
A6003	140S	596	3191
A6003	N12S	156	847
A6900	140S	670	3168
A6900	N12S	113	741

An antiserum prepared against artificial purified OV1 12S antigen had titres against homologous N12S antigen of 1/850 and against homologous 140S antigen of 1/248. This antigen was used in long-fixation CF tests against N12S antigens of the strains OV1 O_1 Lombardy and A6003. The values for $\underline{r}$ obtained from these tests were:

$\underline{r}$ (OV1/O_1Lom) = 0.55

$\underline{r}$ (OV1/A6003) = 0.08

These values were of a similar order to those obtained using the OV1 live-virus antiserum (values of 0.58 and 0.12 respectively). It is probable, therefore, that the presence of large amounts of anti-140S antibody in the live-virus antisera did not influence the results obtained to any large extent; i.e. that the 12S antigens were only reacting with 12S-specific antibody.

An OV1 N12S antigen was tested for potency in long and short fixation tests. The antigen activities obtained were:

short-fixation 407 cfu.,

long-fixation 1709 cfu.

Thus, the potency determined by long fixation was 4.2 times greater than with short fixation.

A preparation of OV1 artificial 12S antigen was compared with natural 12S antigen of the same strain in a long-fixation CF test, by determining the antiserum titres of homologous, sub-type heterologous (O_1 Lombardy) and type-heterologous (A6003) antisera when tested with the two antigens. The results in Table 2.9 show that the naturally-occurring and the artificially-produced antigens were almost identical, with the N12S antigen demonstrating slightly higher titres of all three antisera.

Table 2.9 Titres of OV1, O_1Lom and A6003 antisera, tested with OV1, N12S and A12S antigens

Antigen	Antiserum	Antiserum titre
N12S	OV1	7656
A12S	OV1	7240
N12S	O_1Lom	3734
A12S	O_1Lom	3629
N12S	A6003	60
A12S	A6003	55

2.3 (e) The Type and Sub-type Specificity of VIA Antigens

Preparations of VIA antigen from the type O strains, OV1 and O_1 Lombardy and the type A strain, A6003, were compared for type and sub-type specificity. The OV1 and O_1 Lombardy antigens were tested in long and short-fixation tests (Table 2.10).

Table 2.10 Sub-type relationships between VIA antigens of the strains OV1 and O_1 Lombardy

Fixation period	r_1 (OV1 antiserum)	r_2 (O_1Lom antiserum)	R
long (3 tests)	0.91	0.93	92%
short (2 tests)	0.93	0.92	92%

It can be seen that the antigens were almost identical and that the same relationship (R = 92%) was obtained using both types of CF test. The homologous antiserum titres in these tests were greater in the long-fixation tests by a mean factor of 7.8 compared with those in short-fixation tests.

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A12S	OV1	7240
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Homologous antiserum titres to VIA antigens were much lower than those to either 140S or 12S antigens, as is seen in Table 2.11.

Table 2.11 Homologous antiserum titres to 140S, 12S and VIA antigens

Strain	Antigen	Antiserum titre	
		short-fixation	long-fixation
OV1	140S	3659	13846
OV1	N12S	1163	5435
OV1	VIA	124	878
O_1Lom	140S	1786	6207
O_1Lom	N12S	588	2841
O_1Lom	VIA	87	729

VIA antigens of the OV1 and A6003 strains were compared in a long-fixation test. The relationships obtained were:

r_1 (OV1 antiserum) = 0.87

r_2 (A6003 antiserum) = 0.97

R = 92%

Thus it can be seen that VIA antigens derived from strains of two different types were virtually identical.

2.4 DISCUSSION

The results of the experiments presented in this Section have implications in regard to the structural relationships of the FMD viral capsid and antigenic components derived from it. They also provide information which can be applied to the use of serological tests (in particular, CF) for typing and sub-typing

investigations.

It was found that the intact virion is antigenically completely type specific. This confirms the findings of Brown and Crick (1958) and Bradish and Brooksby (1960). However the method used for this work was more sensitive and could detect slight, spurious cross-reactions which may have been undetectable or mis-interpreted by other methods. Bradish and Brooksby (1960) obtained a low level of heterotypic fixation which they disregarded. Talbot et al. (1973) also demonstrated the type specificity of the 140S antigen. They centrifuged through sucrose density gradients, mixtures of ^{125}I-labelled Fab fragments of the IgG molecule from FMDV antisera and homologous or heterologous virus. A peak of radioactivity, corresponding to the 140S virus peak was obtained only with the homologous antigen.

In contrast to the intact virion, 12S sub-units, both naturally-occurring and artificially-produced, are cross-reactive but still possess considerable type and sub-type specificity. This heterotypic activity was demonstrated by Brown and Crick (1958), and Bradish and Brooksby (1960) indicated that the 12S antigen had some type specificity. However the actual extent of the cross-reactions has not been previously measured.

It was found that the relationship between the N12S antigens of types O and A was closer than those between type O and types SAT 1 and SAT 3. This is possibly a manifestation of a closer relationship between viruses of the European types, than between the European and Southern African types, which is likely, since the Southern African type viruses evolved in geographical isolation from the European ones. However, this suggestion is

made guardedly, since only a small number of cross-comparisons were made.

It was demonstrated that natural and artificial 12S antigens had an almost identical reactivity with homologous, sub-type heterologous and type heterologous antisera. Probably no conclusions can be drawn from the slight differences obtained, since the natural 12S antigens were not purified. They were considered to be free of 140S and VIA antigens, but may have contained other, as yet unidentified, viral antigens.

The polypeptide, VP4, has been shown to react heterotypically (Talbot et al., 1973). However, since the artificially-produced 12S antigen does not possess VP4 (Burroughs et al., 1971; Strohmaier and Adam, 1974), it is evident that there is more than one cross-reacting antigen present in the virus but that it is not reactive when the capsid is intact.

The results of experiments with empty capsids, indicate that they have identical serological specificity to complete virions and this would suggest that the capsid structure of the two kinds of particle is antigenically identical. There are, however, other differences between the particles. As already noted, empty capsids are acid-resistant, SDS-sensitive and relatively unstable. Furthermore, Talbot (1972) demonstrated that empty capsids differed from complete virions in the polypeptide composition shown by polyacrylamide gel electrophoresis. The RNA-free particles possessed VP0 which was interpreted as being a complex of VP2 and VP4 since, with ageing, the proportion of VP0 decreased and that of VP2 and VP4 increased. Strohmaier and Adam (1974) on the other hand, using different conditions for electrophoresis, found that VP0 was normally not detected

and VP4 was never found in empty capsids. Thus the exact nature of the polypeptide differences is not resolved, but it seems reasonable to assume that the capsid structure of the two particles is essentially identical, with minor differences affecting the manner in which they are dissociated under denaturing conditions.

Graves et al. (1968) suggested that empty capsids differed antigenically from complete virions, in reacting with anti-12S serum in agar gel diffusion tests. However, in the light of the present evidence of antigenic identity, it is suggested that the difference found by Graves et al. was due to partial degradation of the less stable empty capsids, exposing antigenic sites which in the intact capsids of full or empty particles are not reactive.

The present findings differ from comparisons made between empty and complete virions of other picornaviruses. Roizman et al. (1958) showed that poliovirus empty capsids consist of a heterogeneous population, some having the same serological reactivity as infectious particles and others being unrelated. Other workers with poliovirus (Scharff et al., 1964; Ghendon and Yakobson, 1971) and with other enteroviruses (Frommhagen, 1965; Forsgren, 1969) have found that complete and empty capsids are serologically unrelated. Lonberg-Holm and Yin (1973) studied complete and empty capsids of human rhinoviruses. They found that the particles possessed some, but not all of their antigens in common. It is possible that the lack of a closer antigenic relationship is the result of relatively minor structural or conformational modifications in the empty capsids of these enteroviruses which, after all, probably contain the

same protein sub-units as the complete virions. Thus the contradictory findings between enteroviruses and FMDV, do not necessarily represent a major difference in the structural relationships of the viral antigens within these two groups.

It has been shown previously by other workers (Rowlands et al., 1971), that trypsin-treated virus has a greatly reduced immunogenicity but that it never-the-less produces significant amounts of neutralising antibody. Brown and Smale (1970) have suggested that the effect of the enzyme is to destroy an antigenic site normally present at regular intervals on the surface of the virion, probably at the icosahedral vertices.

It has been demonstrated in the present work that this antigenic site is highly sub-type specific, since its destruction reduces the homologous antiserum titre but not that of the sub-type heterologous antiserum, when tested against the antigen. This is consistent with previous findings, since it is reasonable to expect that an antigenic site so important for the immunogenicity of the virus would be highly specific.

However, it was also shown that trypsin-treatment only reduced, but did not destroy, the sub-type specific identity of the antigen and this was with supporting evidence that the treated antigen was quantitatively converted to the altered form. This provides convincing evidence that there is more than one sub-type specific antigenic site on the surface of the virion. Since intact virions cross-react between sub-types, there must be at least one other type of surface antigen which is not sub-type specific. Consequently from these experiments with trypsin-treated virus, one can draw the same general conclusion as Brown and Smale (1970); viz. that there appear

to be at least three types of antigenic site on the surface of the virus.

It has been shown that the most cross-reactive antigen in an FMDV harvest is the VIA antigen, which has virtually no type or sub-type specificity. The 12S antigen, although cross-reactive, possesses a considerable degree of specificity. Cowan and Trautman (1967) suggested that VIA antigen was the main cause of cross-reactions encountered in diagnostic CF typing tests and the present work supports this. Consequently a diagnostic CF method for the typing of field strains should be designed to be minimally influenced by VIA antigen in particular and also by 12S antigen.

The CF test currently in use in the World Reference Laboratory for the detection and type differentiation of FMDV in field samples, involves the testing of a single concentration of the unknown antigen against serial dilutions of antisera of each of the seven FMDV types, with a fixed dose ($5C'H_{50}$) of complement. The test is performed in microplates, with primary fixation proceding for 30 minutes at 37°C. The antisera are prepared by the method of Brooksby (1952) (Appendix 3). A number of criticisms can be made of the method and several modifications are proposed.

The diagnostic test should be sensitive in detecting FMDV antigen and selective for type differentiation. The use of a very short fixation test is of relatively low sensitivity and the use of low dilutions of both antigens and antisera predisposes to the detection of cross-reactions. The reaction due to VIA antigen, probably the most important cross-reaction, is also the easiest to exclude. Antiserum produced against inactivated purified 140S antigen does not contain anti-VIA

antibody (Cowan and Graves, 1966). Therefore, the use of such antisera would entirely eliminate VIA cross-reactions.

It is more difficult to avoid cross-reactions due to 12S antigen since anti-140S antisera always possess some anti-12S activity (Cowan, 1968; personal uncited results). However, the homotypic 140S titre of an anti-140S serum should be much greater than the titre to heterotypic 12S antigen. Modification of the test method, to a constant antiserum, varying antigen method, may enable the selection of fixed concentrations of antisera of suitable quality, such that homotypic 140S antigens react with excess antibody but that antibody to heterologous 12S antigen is present in minimal amounts, perhaps undetectable. For example, the OV1 live-virus antiserum used in long fixation tests had a titre of 1/13800 to homologous 140S antigen. The titre for homologous 12S antigen was 3-fold lower and to heterotypic 12S antigens at least 30-fold lower (type A). Therefore, this antiserum could be used at 8 or 16 times the homologous 140S titre and would still not detect heterotypic 12S antigens. Such a test would have the added advantage of being quantitative, the results being expressed as antigen titres. Results of the currently used test, expressed as antiserum titres are quantitatively quite meaningless.

With regard to increasing the sensitivity of the test for antigen detection, it has been shown in this Section that 140S and 12S antigen titres are increased approximately four-fold using a fixation period of 18 hours at 4°C compared with short-fixation (30 minutes at 37°C in tubes, which is equivalent to 60 minutes at 37°C in microplates). Consequently, the use of overnight fixation at 4°C would be expected to increase the

sensitivity of the test by more than four-fold.

Bradish and Brooksby (1960) showed that FMDV antigens cross-reacted between types to a greater extent with long fixation. The present studies of cross-reactions between sub-types suggest that the cross-reactivity of individual antigens does not increase markedly with long fixation. However, while the homologous antiserum titres to 140S and 12S antigens were approximately four times greater in long-fixation tests, the antiserum titres to VIA antigens increased eight-fold. Consequently, the marked degree of increased cross-reactivity observed by Bradish and Brooksby (1960), was possibly due to a more selective measurement of VIA antigen. It follows from the present results that the use of a long-fixation test, if performed with the other modifications proposed to minimise cross-reactions, would increase the sensitivity without markedly decreasing the specificity of the test.

It is also possible to make certain observations regarding the procedure for sub-type differentiation. It was demonstrated that 140S, 12S and VIA antigens have different sub-type specificities, the 140S antigen being the most specific. In contradistinction to type differentiation, which requires sensitivity of antigen detection and type but not sub-type specificity, for sub-type differentiation the most important requirement is for specificity of the reaction, so that only the 140S antigen/antibody reaction is being measured.

In Section 1, it was shown that CF tests for sub-type differentiation used by many earlier workers were quantitatively invalid. It is also probable that the use of a test with a constant concentration (in so-called excess) of crude antigen

means that cross-reactions are being measured between the three complement-fixing antigens, to an extent depending on their relative concentrations and the relative concentrations of their antibodies in the antisera.

It is evident from results given in this Section and also from the results of Cowan and Trautman (1967), that antisera produced in guinea pigs using live virus as antigen, are most reactive with 14OS antigen, less so with 12S antigen and even less with VIA antigen. Consequently, the method developed in Section 1 for sub-type differentiation, being based on dilution of the antisera to their end-points, will tend to measure the 14OS reaction in preference to the others. The use of purified 14OS antigen will ensure that there is no VIA antigen and little 12S antigen present, although a certain degree of breakdown of the 14OS antigen is inevitable. The evidence from tests using 12S-adsorbed antisera, with which sub-type specificity was the same as with unadsorbed antisera, suggests that with the procedure used for these tests, only the reaction of 14OS antigen was being measured. Further evidence for this is provided in Section 3, from the results of cross-protection tests and kinetic serum neutralisation tests.

When the CV1 and O_1 Lombardy strains were compared for sub-type specificity using anti-14OS sera, the results were very similar to those obtained with live-virus antisera. Differences may be attributable to different avidities of the antisera and this aspect is discussed further in Section 3.4.

It has been shown previously (Bradish and Brooksby, 1960) and confirmed in this study, that the reaction with 14OS antigen is more specific in a short-fixation than in a long-fixation test.

Therefore it is an advantage to use a short-fixation period (30 minutes at 37°C in tubes or 60 minutes at 37°C in microplates) for greater sensitivity in sub-type differentiation.

2.5 CONCLUSIONS

The intact virion has the greatest sub-type specificity of the FMDV antigens and cross-reactions between sub-types are less evident in short-fixation tests. Fresh preparations of this antigen have no heterotypic reactivity.

12S sub-units, both naturally-occurring and artificially-produced are cross-reactive but still possess considerable type and sub-type specificity. In contrast, the non-structural virus infection-associated antigen is almost completely non-specific with regard to type and to sub-type.

Empty capsids appear to have a sub-type specificity and antigenic potency identical to that of intact virions. Trypsin-treated 140S antigens had a reduced reactivity with their homologous antisera and a lower sub-type specificity. This verified that the polypeptide, VP1, which is altered by the action of trypsin, is associated with a highly specific antigenic site.

A number of recommendations were made, for the type identification of field isolates of FMDV by CF. Tests should be carried out with a long fixation period for maximum sensitivity of antigen detection. Antisera should be prepared against inactivated antigens, to eliminate cross-reactions due to the presence of VIA antigen. A titration of the unknown antigen against constant concentrations of known antisera, would provide a quantitative result.

It was verified that the method proposed in Section 1 for the sub-type differentiation of FMDV strains is a suitable

method for the detection of strain differences. The use of a short-fixation test and the determination of antiserum end-points favours the measurement of the 140S reaction.

SECTION 3. AN EVALUATION OF THE SIGNIFICANCE OF FMDV STRAIN DIFFERENTIATION BY COMPLEMENT-FIXATION

3.1 INTRODUCTION

The detection of antigenic differences between FMDV strains within a type has received considerable attention because of its importance in the field, especially in relation to the identification of new strains and the selection of vaccine strains. From this aspect, the final criterion for the differentiation of two strains should be their performance in cross-protection tests in livestock. However, such tests are likely to be prohibitively expensive and other methods have been sought. CF has become the most widely applied technique for strain differentiation but, for its application to be valid, it must have a reasonable correlation with differences determined by cross-protection.

Traub and Möhlmann (1946) showed that strains which could be differentiated by CF were also immunogenically different, in that groups of cattle vaccinated with one strain withstood homologous challenge but were only partially protected against challenge with a heterologous strain. Similar qualitative results were also obtained by a number of other workers (Henderson *et al.*, 1948; Martin *et al.*, 1962; Hyslop *et al.*, 1963; Hedger and Herniman, 1966). Several of them (Brooksby *et al.* 1948 a & b; Hyslop *et al.* 1963; Hedger and Herniman, 1966) have shown that antigenic differences detected by CF and by cross-protection tests can also be demonstrated by cross-serum neutralisation tests.

Quantitative cross-protection tests have also been devised, based on the determination of heterologous and homologous 50%

protective dose (PD_{50}) values. Muntiu (1965) described the use of such a test in cattle and found that the difference obtained between two strains was similar to that determined by CF. Fontaine et al. (1966) developed a similar cross-protection test for FMDV strain differentiation using guinea pigs and this method has been used by other workers (Moosbrugger et al. 1967; Tekerlekov, 1973). However, there is very little comparative evidence by which to assess the significance of differences detected by serological tests in terms of the susceptibility of vaccinated animals to challenge by heterologous strains in the field.

In this Section are described comparative tests carried out with two FMDV strains to demonstrate differences by complement-fixation, by kinetic serum neutralisation tests, by conventional cross-neutralisation tests and by a guinea pig cross-protection test.

The antigenic differentiation of strains by CF has been used as the basis for the classification of strains within a type into sub-type groups. Such a classification should demonstrate the range of antigenic variation that occurs within a type and should simplify the procedure for the identification of new strains. If all known strains can be placed in sub-type groups, then a small number of comparisons with an unknown strain should enable one to classify the new strain and so obtain some indication of its relationship to all other strains.

The term "sub-type" has never been precisely defined. Brooksby (1968) proposed it as follows: "A sub-type can then be defined as a group of strains which can be differentiated from other groups of strains within the type by serological

methods or by cross immunity experiments at a level of immunity lower than that possessed by recently recovered animals - for example, in vaccinated animals." The implication is that animals vaccinated with one strain would be protected against challenge with another strain of the same sub-type, but not of a different sub-type. Brooksby (1968) suggested the following values for R (as defined in Section 1.2) for type and sub-type differentiation by CF:

(1)	type difference	R = 10% or lower
(2)	sub-type widely different	R = 10% - 32%
(3)	sub-types different	R = 32% - 70%
(4)	differences within sub-type	R = 70% - 100%

Following these criteria, the World Reference Laboratory at Pirbright has classified strains of each type into a large number of sub-type groups - from 3 sub-types within the Asia 1 type to 32 sub-types within type A.

If the genetic alteration involved in the formation of a new sub-type occurred as a single mutation which was readily detectable antigenically, then sub-type classification would be straightforward with each strain falling into one or another group. However, if the alteration was the result of a number of small antigenic changes, then there would be no natural separation into groups and the selection of sub-type limits must be somewhat arbitrary. The selection of such limits is reasonable if it is useful. To classify all strains as one sub-type or, alternatively, to classify every antigenic variant as a new sub-type would be of little benefit. Consequently, it is important to determine both the character and the range of antigenic variation within a type, to satisfactorily classify

strains. In this Section, the results are presented of a large number of cross-CF tests carried out between strains of one type, to obtain an indication of the antigenic variation encountered within a type. On the basis of these results, new criteria are proposed for the classification of sub-types of FMDV.

3.2 MATERIALS AND METHODS

3.2 (a) The Guinea Pig Protection Test

Viruses were grown on BHK monolayers, inactivated with AEI and purified by sucrose density gradient centrifugation all as described in Appendices 2 (a) and (b). The gradient fractions containing the peaks of 140S activity were identified by CF assay (Appendix 4). The quantity of virus present in each peak fraction was estimated by measuring the OD of the sample at 259 nm. and calculating the concentration of virus on the basis of the finding of Bachrach *et al.* (1964) that 1 OD unit = 132 µg./ml. of purified virus.

Vaccines were prepared in three-fold dilution series by suitably diluting the virus in 0.04M phosphate buffer at pH 7.6, containing 0.1% bovine albumin powder and 0.01% saponin. Guinea pigs of approximately 600 g. bodyweight were inoculated subcutaneously with a 1 ml. dose of vaccine, on the day following the purification of the viruses. For each dilution of each vaccine, groups of eight guinea pigs were inoculated for homologous or heterologous challenge.

Viruses for the challenge of vaccinated guinea pigs were prepared as described in Appendix 2 (a) and titrated for infectivity by inoculation of virus in 10-fold dilution series from the guinea pig adapted stock virus. 0.1 ml. of each dilution was inoculated intradermally into one tarsal pad of each of five

guinea pigs. The animals were examined daily for seven days and where specific lesions of FMD were observed at sites other than the inoculation site, a positive result was recorded. The infectivity of the stock viruses was then calculated by the method of Kärber (1931).

At 28 days after vaccination, groups of guinea pigs were challenged with the homologous or heterologous virus by inoculating them with an estimated $10^{2.0}$ guinea pig 50% infective doses ($GPID_{50}$) in the same manner as for the challenge virus titrations and positive reactions were assessed by the same criteria. The challenge viruses were re-titrated at the same time.

From the results of the test, the 50% protective dose (PD_{50}) was calculated for each vaccine for homologous and heterologous challenge by the method of Reed and Meunch (1938).

A value for $\underline{r}$ was determined for each vaccine, being the proportion PD_{50} (homologous)/PD_{50} (heterologous). From values of $\underline{r}_1$ and $\underline{r}_2$ for the two vaccines, the cross-protection relationship, $\underline{R}$ (CP), was determined for the two viruses in a manner analogous to that described in Section 1.2 for the CF test.

At 21 days after vaccination, blood samples were taken from every animal by cardiac puncture. Assays for serum neutralising antibody activity were carried out in microplates as described in Appendix 8. Sera from all guinea pigs vaccinated with the same strain of virus were assayed in the same test. They were diluted in the plates from a starting dilution of 1/4 and were tested against an estimated $10^{1.5}$ $TCID_{50}$ of homologous virus per well.

3.2 (b) The Kinetic Neutralisation Test

Kinetic neutralisation tests were performed by mixing virus and antiserum and taking samples, at fixed time intervals, which

were immediately diluted and subsequently titrated for plaque production in tissue culture.

The diluent used was phosphate-buffered saline, pH 7.4, with 0.1% bovine albumin powder (PBSA). The BHK adapted, glycerinated viruses (Appendix 2 (a)) were diluted to an infectivity of between 3×10^5 and 8×10^5 pfu./ml. Guinea pig antisera were inactivated at 56°C for 30 minutes and diluted to a predetermined concentration which would give a suitable rate of neutralisation.

Neutralisation was carried out at room temperature. Two viruses were tested against one antiserum in the same test. One ml. of virus was added to 1 ml. of PBSA (to determine the initial infectivity) or to 1 ml. of antiserum. Samples of 0.25 ml. were taken from the virus/antiserum mixtures at fixed intervals and mixed with 24 ml. of diluent at 0°C. Three-fold dilutions were then made from these and each dilution used to inoculate three IB-RS-2 monolayers in Petri plates (Appendix 6). After incubation, fixing and staining (Appendix6), plaques were counted and the mean titre for each sample was determined from a weighted mean of dilutions producing between 10 and 100 plaques on each plate.

For each virus/antiserum mixture, the rate of neutralisation was determined by the method of least squares, from the infectivity titres of individual samples. A neutralisation constant (K) was then determined using the equation:

$$K = D/t \times 2.3 \log_{10} Vo/Vt,$$

where D = reciprocal of the antiserum dilution,

t = time in minutes and

Vo and Vt represent the infectivity of the mixture at times O and t respectively. Using the values of K for homologous

and heterologous reactions obtained in the same test, values for $\underline{r}$ were determined for each antiserum, being the proportion K (heterologous)/K (homologous). With values of $\underline{r}_1$ and $\underline{r}_2$ for the two antisera, the kinetic neutralisation relationship, $\underline{R}$(KN), was determined for the two viruses as previously described (Section 1.2).

3.2 (c) The Conventional Cross Serum Neutralisation Test

Cross-serum neutralisation tests were carried out in microplates. The reagents used and the conditions are described in Appendix 8.

To compare two strains, each antiserum was diluted in two-fold intervals and titrated in eight replicates against two concentrations each of homologous and heterologous viruses, estimated to be $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$ per well. Antiserum titres for each concentration of each challenge virus were determined from the test and the titre of each antiserum against $10^{2.0}$ $TCID_{50}$ of each virus was determined by graphical interpolation between the titres for the two challenge doses. Values of $\underline{r}$ for each antiserum were determined as the proportion of $\frac{\text{titre with heterologous virus}}{\text{titre with homologous virus}}$. From values for $\underline{r}_1$ and $\underline{r}_2$ a value for the cross-neutralisation relationship, $\underline{R}$(CN), was determined as described in Section 1.2.

3.2 (d) Complement-fixation Tests in Microplates for Sub-type Differentiation

The differentiation of strains by CF was carried out using the principles developed in Section 1. However, a number of modifications were made to enable a large number of tests to be carried out on a routine basis.

Unless otherwise stated, the antigens used were BHK monolayer harvests, concentrated and partially purified by pelleting as described in Appendix 2 (b), but without the final purification step of sucrose density gradient centrifugation. These are referred to as pelleted 140S antigens. Antigens were stored at -70°C, diluted in VBS. The results of many tests with antigens used before and after storage, indicated that there was no significant alteration to the antigens on storage for up to three months.

Guinea pig antisera were prepared by two methods. Live-virus antiserum was prepared using guinea pig adapted virus as described by Brooksby (1952) (Appendix 3 (a)). 140S antiserum was prepared using purified, inactivated 140S antigen as described in Appendix 3 (c). The latter method enabled antisera to be prepared in a shorter time and without the need for high security animal accommodation.

The cross-CF tests were carried out in microplates as described in Section 1.2.

3.3 RESULTS

3.3 (a) The Guinea Pig Protection Test

The two type A strains, A6003 and A6900, previously compared by CF (Section 1.3 (b)) were compared in a guinea pig cross-protection test. Purified, inactivated 140S antigen of the two strains contained 300 µg./ml. (strain A6003) and 250 µg./ml. (strain A6900) of virus in the peak fractions of the preparative sucrose density gradients.

Guinea pigs were challenged 28 days after vaccination with an estimated $10^{2.0}$ $GPID_{50}$ of either the homologous or the heterologous virus. The actual infectivity titres of the two challenge

viruses, from titrations at the time of challenge were $10^{1.9}$ (A6003) and $10^{2.1}$ (A6900) $GPID_{50}$. The results are shown in Table 3.1.

From the PD_{50} values, the values for $\underline{r}$ and $\underline{R}$ (CP) were calculated and are as follows:

$\underline{r}_1$ (vaccine A6003) = 0.33

$\underline{r}_2$ (vaccine A6900) = 0.36

$\underline{R}$(CP) = 35%

The results of titrations for neutralising antibody in sera obtained at 21 days after vaccination are shown in Table 3.2. It is evident that the antibody titres were very low and in fact, around the level of 1 PD_{50} (homologous) for each vaccine, antibody was generally not detectable. However, the method of antibody titration is considered to be relatively sensitive. For comparison, reference antisera were titrated in the same tests, being those used for the CF tests in Section 1.3 (b). Their antibody titres were 1/4096 (A6003) and 1/3388 (A6900).

Although serum antibody levels were mainly undetectable for vaccine doses around the minimum for homologous and heterologous protection, the mean antibody titres for groups of guinea pigs vaccinated with larger doses of antigen suggest that there was a decrease in antibody titre of the order of two-fold for each three-fold decrease in antigen dose. If it is assumed that a relationship of a similar order would hold for smaller vaccine doses around the 1 PD_{50} level, then it can be argued that for these two strains, heterologous protection requires about twice the concentration of circulating antibody needed for homologous protection.

Table 3.1. Results of homologous and heterologous challenge of guinea pigs vaccinated with purified 140S antigen of FMDV strains A6003 and A6900

Challenge virus strain	Vaccine virus strain	Antigen dose (ng)	Proportion of positive reactions	Value of PD_{50} (ng)
A6003	A6003	1,111	0/8	33
		370	0/8	
		123	0/8	
		41	3/8	
		14	7/7	
A6900	A6003	10,000	0/8	99
		3,333	0/8	
		1,111	0/8	
		370	0/8	
		123	3/8	
		41	8/8	
		14	7/8	
A6900	A6900	1,111	0/8	90
		370	0/8	
		123	4/7	
		41	5/8	
		14	8/8	
A6003	A6900	10,000	0/7	258
		3,333	0/8	
		1,111	0/8	
		370	2/8	
		123	7/7	
		41	7/8	
		14	8/8	

Table 3.2 Neutralising antibody titres of sera from guinea pigs 21 days after vaccination with purified virus vaccines of the strains A6003 and A6900

Vaccine virus strain	Antigen dose (ng)	Neutralising antibody titres		Proportion with positive titres
		Range	Mean	
A6003	10,000	32-128	69	8/8
	3,333	16-45	30	8/8
	1,111	11-45	25	16/16
	370	4-45	11	16/16
	123	<4-16		7/15
	41	<4-8		2/16
	14	<4-4		1/15
A6900	10,000	32-128	59	8/8
	3,333	45-128	76	8/8
	1,111	11-181	36	16/16
	370	8-64	18	15/15
	123	<4-45		8/15
	41	<4-8		2/15
	14	<4-4		1/15

3.3 (b) The Kinetic Neutralisation Test

Kinetic neutralisation tests were carried out with the two type A strains, A6003 and A6900, which had been compared by CF (Section 1.3 (b)) and by cross-protection in guinea pigs (Section 3.3 (a)). The antisera were those used for the CF tests in Section 1.3 (b).

Preliminary experiments were carried out to verify that the rate of neutralisation in this system is determined by the principles elucidated by Dulbecco et al. (1965), on which the validity of the formula used for the calculation of K-values is dependent.

An experiment carried out with A6900 virus and antiserum showed that with a virus concentration of approximately 3×10^5 pfu./ml. and antiserum diluted 1/1000, the reaction appeared to be of the first order for a period of about 10 minutes (Figure 3.1). To ensure that neutralisation was being measured during the period of first order reaction, subsequent experiments were carried out over four minutes, taking samples at one minute intervals.

A further experiment with A6900 virus and antiserum (Figure 3.2) demonstrated that the first order character of the reaction was independent of antiserum concentration. The values of K for the three antiserum dilutions were:

antiserum 1/1600, K = 276;

antiserum 1/800, K = 350;

antiserum 1/400, K = 299.

These values determined for K can be considered the same (within experimental error) and this indicates that the value of K is independent of the antiserum concentration. Consequently the

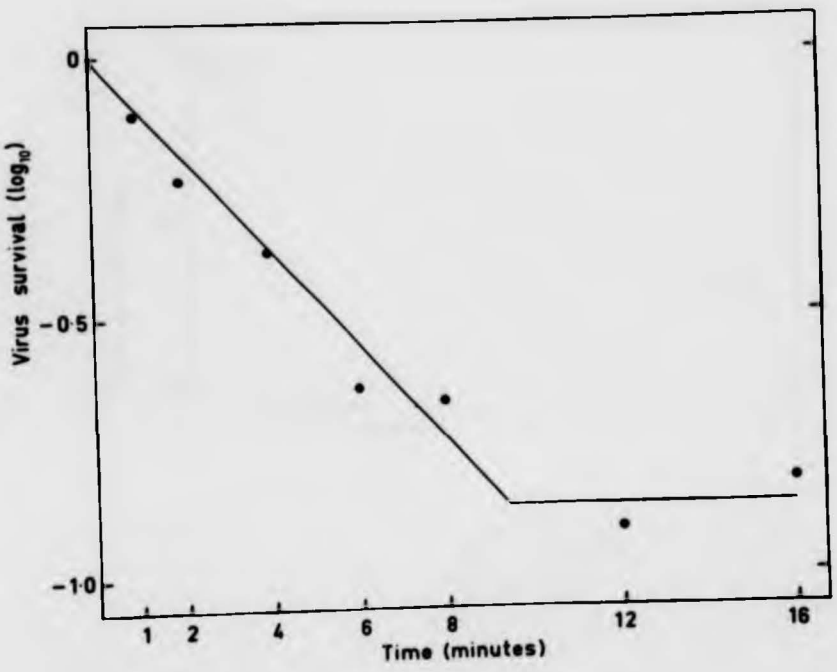

Figure 3.1. Kinetic curve of neutralisation of A6003 virus by homologous antiserum.

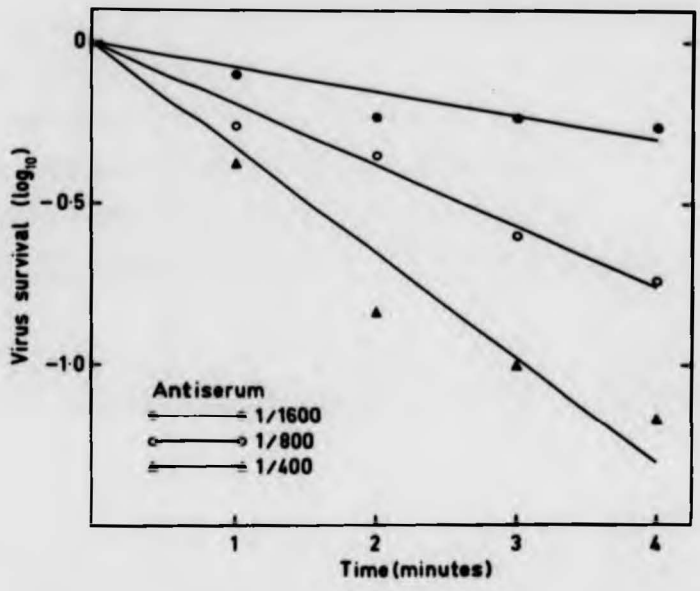

Figure 3.2. Kinetic curves of neutralisation of A6003 virus by varying dilutions of homologous antiserum.

rate of neutralisation was proportional to the concentration of antiserum.

Three experiments were then performed with both of the antisera, A6003 and A6900, to measure the values of K for homologous and heterologous reactions and from these to determine values for r and R(KN). Figure 3.3 shows the results of typical experiments with each antiserum. The values of K and r, determined from the experiments, are presented in Table 3.3.

Table 3.3 Values for K and r, determined by kinetic neutralisation tests with the strains A6003 and A6900

Antiserum	Expt. No.	K (homologous)	K (heterologous)	r
A6003	1	171	49.6	0.29
A6003	2	154	70.7	0.46
A6003	3	152	53.5	0.35
A6003	4	124	50.0	0.40
A6900	1	161	94.3	0.59
A6900	2	271	161	0.59
A6900	3	219	117	0.53

It is apparent from Table 3.3 that although individual K values were highly variable, the values of r were rather less so (the maximum range of variation was $\pm$ 25% of the mean value). From the values for r, mean values for r_1, r_2 and R(KN) were obtained as follows:

r_1 (antiserum A6003) = 0.37

r_2 (antiserum A6900) = 0.56

R(KN) = 46%

rate of neutralisation was proportional to the concentration of antiserum.

Three experiments were then performed with both of the antisera, A6003 and A6900, to measure the values of K for homologous and heterologous reactions and from these to determine values for $\underline{r}$ and $\underline{R}$(KN). Figure 3.3 shows the results of typical experiments with each antiserum. The values of K and $\underline{r}$, determined from the experiments, are presented in Table 3.3.

Table 3.3 Values for K and r, determined by kinetic neutralisation tests with the strains A6003 and A6900

Antiserum	Expt. No.	K (homologous)	K (heterologous)	$\underline{r}$
A6003	1	171	49.6	0.29
A6003	2	154	70.7	0.46
A6003	3	152	53.5	0.35
A6003	4	124	50.0	0.40
A6900	1	161	94.3	0.59
A6900	2	271	161	0.59
A6900	3	219	117	0.53

It is apparent from Table 3.3 that although individual K values were highly variable, the values of $\underline{r}$ were rather less so (the maximum range of variation was $\pm$ 25% of the mean value). From the values for $\underline{r}$, mean values for $\underline{r}_1$, $\underline{r}_2$ and $\underline{R}$(KN) were obtained as follows:

$\underline{r}_1$ (antiserum A6003) = 0.37

$\underline{r}_2$ (antiserum A6900) = 0.56

$\underline{R}$(KN) = 46%

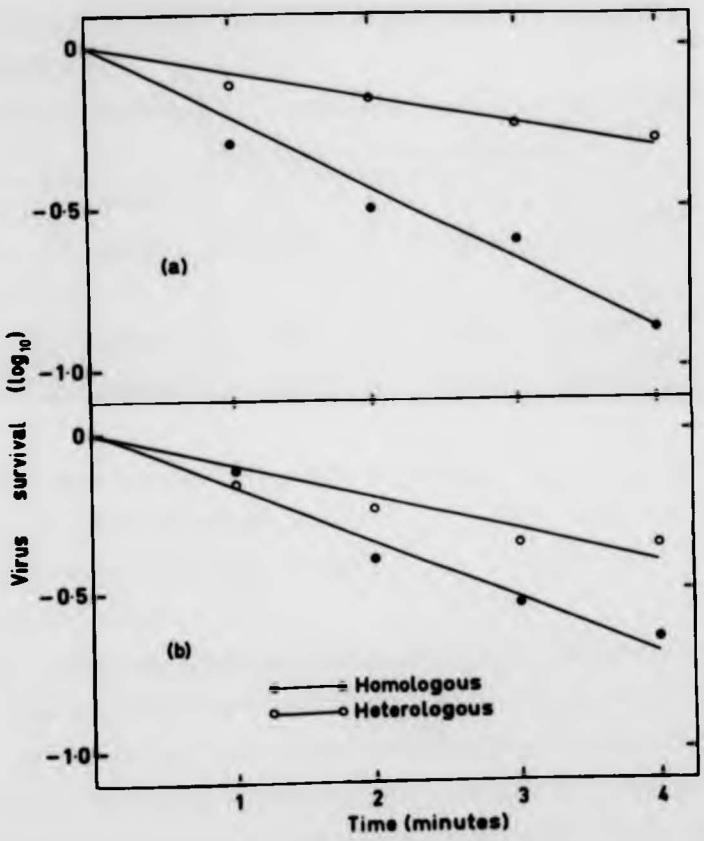

Figure 3.3. Kinetic curves of neutralisation of (a) A6003 virus and (b) A6900 virus by homologous and heterologous antisera.

3.3 (c) The Conventional Cross Serum Neutralisation Test

Cross serum neutralisation tests were carried out with the strains A6003 and A6900. Viruses and antisera were the same as those used for the kinetic neutralisation tests. Two tests were carried out and the results are shown in Table 3.4.

Table 3.4 Cross serum neutralisation tests with strains A6003 and A6900

Expt. No.	Antiserum	Antiserum titre		r	R(CN)
		homologous	heterologous		
1	A6003	1202	513	0.43	33%
	A6900	3236	851	0.26	
2	A6003	1479	457	0.31	30%
	A6900	2427	692	0.29	

The mean values from Table 3.4 are:

r_1 (A6003) antiserum = 0.36

r_2 (A6900) antiserum = 0.27

R(CN) = 32%

3.3 (d) Cross-CF Tests with Strains of FMDV Type SAT 1

Cross-CF tests were carried out with 16 strains of type SAT 1 FMDV. It was desired to investigate the range and the pattern of variation between strains, in order to evaluate the validity of criteria used for sub-type classification. The strains included one of each sub-type as classified by the World Reference Laboratory and a number of recent field isolates from West, East and Southern African countries.

Some antisera were obtained from the World Reference Laboratory and had been prepared by the method of Brooksby (1952)

(live-virus antisera). Others were prepared against purified, inactivated 140S antigens (140S antisera). Unless otherwise specified, the antisera used were as follows:

RV 11/37 (Rhodesia, 1937),	140S
SWA 1/49 (South West Africa, 1949),	live virus
SR 2/58 (Southern Rhodesia, 1958),	live virus
SA 13/61 (South Africa, 1961),	live virus
SWA 40/61 (South West Africa, 1961),	live virus
TUR 323/62 (Turkey, 1962),	140S
GHA 14/68 (Ghana, 1968),	live virus
BOT 1/68 (Botswana, 1968),	140S
MAL 3/70 (Malawi, 1970),	140S
UGA 47/71 (Uganda, 1971),	140S
KEN 3/72 (Kenya, 1972),	140S
RHO 4/72 (Rhodesia, 1972),	140S
ANG 9/72 (Angola, 1972),	140S
NIG 9/72 (Nigeria, 1972),	140S
GHA 7/73 (Ghana, 1973),	140S
ZAM 25/73 (Zambia, 1973),	140S

The first six strains above, represent the six sub-types currently recognised by the World Reference Laboratory.

The results of the tests are summarised in Table 3.5. The values for R represent geometric mean values of at least two tests. If individual values for R between two strains differed by two-fold or more, further tests were carried out.

The range of values of R shown in the Table is large and suggests a continuous gradation from virtual identity (90%) to 2% or less. There is no clear pattern of variation, in that strains which are furthest apart in time of isolation are not

Table 3.5 Values for R (%) determined by cross-CF between strains of FMDV type SAT 1

	RV11/37	SWA1/49	SR2/58	SA13/61	SWA40/61	TUR323/62	GHA14/68	BOT1/68	MAL3/70	UGA47/71	KEN3/72	RHO4/72	ANG9/72	NIG9/72	GHA7/73	ZAM25/73
RV11/37		30	27	16	23	9	3	14	4	10		15	<3		9	19
SWA1/49			23	35	32	21	7									
SR2/58				27	55	32			14							
SA13/61					42	24	10		21			23	10		18	25
SWA40/61						32	<6									
TUR323/62							22	33	21	30	43	23	11		10	10
GHA14/68									4			5	<3		5	<3
BOT1/68									28	31	30	16	7		7	15
MAL3/70										18	96	16	13		4	25
UGA47/71											14				3	
KEN3/72																
RHO4/72													11	11	12	23
ANG9/72															2	4
NIG9/72															65	
GHA7/73																3

necessarily those showing the greatest differences. Also there is no indication that the strains can be readily classified into groups of related strains. This problem is discussed at length in Section 3.4 (b).

Since the antisera used were prepared by two different methods, it was considered necessary to make some comparison of their quality. Live-virus and 140S antisera of the strains RV 11/37 and BOT 1/68 were tested against pelleted antigens, purified 140S antigens and 12S antigens (prepared by heating purified 140S antigen at 56°C for 30 minutes). Table 3.6 shows the results of cross-CF tests and in Table 3.7, antiserum titres to homologous 140S and 12S antigens are shown. All results represent mean values from at least two tests.

It is evident from Table 3.6, that the values determined for r and R were similar with pelleted or purified 140S antigens but different with 12S antigens. This suggests that the pelleted antigens were of good quality; i.e. that they consisted mainly of 140S antigen. However, the values determined with either pelleted or purified 140S antigens were very different, depending on the type of antisera used. The homologous titres (Table 3.7) of three of the four antisera were greater with 140S than with 12S antigens. However, for the BOT 1/68 live-virus antiserum, the anti-12S titre was greater than the anti-140S titre. The relative anti-12S and anti-140S titres of the sera do not appear to fully explain the greater specificity of the two 140S antisera compared with the live-virus antisera, since for RV 11/37, the two antisera were similar in this respect but the value of r for the anti-140S serum was lower than that for the live-virus antiserum. Nor do they explain the lower value for R obtained with

Table 3.6 Cross-CF tests using live-virus and 140S antisera, with pelleted, purified 140S and 12S antigens

Antigens	Antisera	r_1 (RV11/37)	r_2 (BOT1/68)	R
pelleted	live-virus	0.30	0.71	46%
pelleted	140S	0.12	0.25	18%
purified 140S	live-virus	0.25	0.85	47%
purified 140S	140S	0.06	0.25	12%
12S	live-virus	0.30	0.35	33%
12S	140S	0.30	0.25	30%

Table 3.7 Antiserum titres against homologous purified 140S and 12S antigens

Antigen	Antiserum	Antiserum titre
140S	RV 11/37 live-virus	204
12S	RV 11/37 live-virus	128
140S	RV 11/37 140S	204
12S	RV 11/37 140S	181
140S	BOT 1/68 live-virus	115
12S	BOT 1/68 live-virus	181
140S	BOT 1/68 140S	724
12S	BOT 1/68 140S	181

the live-virus antisera, when tested against 12S antigens, compared with pelleted or purified 140S antigens (Table 3.6). These anomalies were not resolved. However, it indicates that the results of cross-CF tests with antisera which have not been fully characterised, must be interpreted with considerable care. The implications of this are discussed further in Section 3.4 (b).

3.4 DISCUSSION

3.4 (a) Comparison of the CF Test with other Immunological Methods for Strain Differentiation

In this Section, an attempt has been made to provide some evidence that the differences between strains as detected by CF can be correlated to those differences which are detected on the basis of cross-neutralisation or cross-protection. It would appear reasonable to assume that if the differences determined by CF, by neutralisation *in vitro* and by protection *in vivo* are of a similar magnitude then in each system the same combinations of antigen and antibody are being detected.

For the two strains, A6003 and A6900, the relationships determined by CF (short-fixation in tubes; from Section 1.3 (b)), by conventional cross-neutralisation, by kinetic neutralisation and by animal cross-protection were expressed as values for $\underline{R}$, $\underline{R}$(CN), $\underline{R}$(KN), and $\underline{R}$(CP) respectively. These relationships are comparable in being derived in the same manner from values for $\underline{r}$, which in each case were determined as the proportion of heterologous reaction/homologous reaction, for two antigens reacting with one antiserum (or, in the case of cross-protection, two viruses challenging guinea pigs inoculated with one vaccine). The values for $\underline{R}$, $\underline{R}$(CN), $\underline{R}$(KN) and $\underline{R}$(CP) were respectively, 37%, 32%, 46% and 35%. Since they are all of a similar order, this

provides some support for the validity of using CF to determine the relationships between strains. It is recognised that further comparisons of a similar nature would be desirable but these must inevitably be limited by the high cost of large-scale animal experiments.

It is evident that the guinea pig protection test described in this Section is an effective method for the detection of strain differences. Animal protection tests suffer from the disadvantages of being costly and time-consuming and of being subject to considerable variation due to differences in individual animal susceptibility. Their greatest advantage is that differences determined by animal protection are closely analogous to the situation for which the information is required; viz., the susceptibility of vaccinated animals to challenge in the field. The results obtained in the test described in Section 3.3 (a), suggest that variation in individual animal susceptibility was not an important source of error, since all end-points were clearly defined over a range of only one or two vaccine dilutions.

The use of vaccines prepared from purified 140S antigens for cross-protection tests has not been previously described. Nor has the response of guinea pigs to challenge after vaccination with such preparations. Mowat (1972) determined the PD_{50} for pigs and cattle of purified 140S antigen vaccines prepared from strains of types A, SAT 2 and Asia 1. For pigs, the PD_{50} values ranged from 5.8 ng. to 14.1 ng., depending on the strain and for cattle, from 32 ng. to 255 ng. The present results suggest that guinea pigs require similar doses of 140S antigen to cattle, for protection against homologous challenge.

Morgan *et al.* (1970) vaccinated guinea pigs with purified

140S antigen and measured the development of neutralising antibody in their sera. They found that only guinea pigs inoculated with more than 160 ng. of antigen, had detectable levels of neutralising antibody 28 days after vaccination. The present results confirm these findings.

Neutralising kinetics have been used for strain differentiation within many virus groups; for example, picornaviruses (McBride, 1959; Richter, 1972), influenza viruses (Pereira and Tumova, 1967), reoviruses (Munro and Wooley, 1973) and poxviruses (Dunlap and Barker, 1973). Capstick et al. (1959) studied neutralisation kinetics with FMDV but the method has not previously been applied to FMDV strain differentiation. The method was shown to be reasonably reproducible and to demonstrate differences between two strains to a similar extent to CF. From the results of the present work, it would appear that the kinetic neutralisation test has no advantage over the CF test for sensitivity or reproducibility. However, it could prove to be a useful method for the detection of strain differences where antisera suitable for CF are not available.

The conventional cross serum neutralisation test performed in microplates also appeared to provide a satisfactory differentiation between the two strains. It is a simple and rapid technique and for this reason could have a greater application than the kinetic test as described in this Section. For both of these neutralisation tests, a more extensive evaluation is required, before their reproducibility and their usefulness for FMDV strain differentiation can be fully assessed.

The reasonably close agreement between the results of the cross-protection test, the CF test and the cross-neutralisation

tests, support the conclusions of the other workers referred to earlier (Section 3.1). However, as previously pointed out, most of the earlier work was not carried out on a quantitative basis. Guerche *et al.* (1972) suggested that strains which were distinguishable in cross-protection tests, could not be differentiated by CF. However, since their two strains, A6003 and A6900, were used for the present work in which they were clearly differentiated by both methods, their conclusion is refuted.

3.4 (b) The Sub-type Classification of FMDV Strains

From the results of cross-CF tests carried out with SAT 1 strains, it is apparent that the antigenic variation between strains (as expressed in values for R) is greater than has previously been recognised. As discussed in Section 2.4, it is likely that the CF test used by previous workers for FMDV strain differentiation, tended to mask differences between strains by enabling antigens of low sub-type specificity to interfere with the results. Also, it has been shown in this Section that antisera prepared using live virus may be inferior for distinguishing antigenic differences. On the other hand, it was found that pelleted antigens were as good as purified 140S antigens for determining strain differences.

The results obtained from the CF tests between SAT 1 strains were not all strictly comparable, due to the two types of antisera used. However, a sufficient number of results were obtained using anti-140S sera to suggest that they give a real indication of the degree of antigenic variation that can be encountered within a type.

Brooksby (1968) suggested a value of R = 10% as representing the greatest difference that occurs between strains of the same

type. The results in this Section demonstrate that differences can be much greater than this (down to as low as 2%). It can also be seen that if $\underline{R}$ = 70% was the smallest difference separating sub-types, then almost all of the strains examined would be classified as different sub-types. This would provide little information regarding the inter-relationships between the strains. It is suggested that a more realistic level for sub-type differentiation would be $\underline{R}$ = 25%, which represents a four-fold difference between the homologous and heterologous titres of the antisera of the two strains being compared. It is only around this level of differentiation that differences detected in microplate tests become meaningful, the detection of smaller differences requiring more accurate techniques (such as the tube test described in Section 1).

Examination of the relationships obtained for the SAT 1 strains (Table 3.5), reveals that these strains cannot be placed into groups based on a level of differentiation of $\underline{R}$ = 25%, such that all strains within a group are more closely related to each other than to strains in other groups. It follows that these strains cannot be classified in this way, so as to fulfil the conditions of Brooksby's (1968) definition. Consequently a new approach to sub-type classification is suggested.

Since all strains cannot be uniquely classified, it is proposed to strictly classify only <u>reference strains</u>, one for each sub-type, such that the relationship of one reference strain to all other reference strains is $\underline{R} < 25\%$. The selection of reference strains with incomplete historical data must be somewhat arbitrary but has a chronological basis. Once selected, reference strains are fixed. All other strains are classified

as related strains, in all sub-type groups with whose reference strain they have a relationship of $R > 25\%$. From the results obtained with the SAT 1 strains (Table 3.5) a sub-type classification can be made as shown in Table 3.8.

The system may appear to increase the complexity of sub-type classification. However, if the concept of sub-types as presently accepted (Brooksby, 1968) is to be retained at all, then it must be in such a form as to allow this flexibility in classifying related strains. The exhaustive characterisation of related strains is not essential as the division into sub-types depends only on the identification of reference strains. The six strains presently classified in the World Reference Laboratory as separate sub-types, are all contained in the first three sub-type groups in Table 3.8. The identification of a further six groups demonstrates the antigenic diversity of SAT 1 strains which have not been previously classified.

Table 3.8 Sub-type classification of SAT 1 strains

Sub-type	Reference strain	Related strains
SAT 1/1	RV 11/37	SWA 1/49, SR 2/58
SAT 1/2	SA 13/61	SWA 1/49, SR 2/58, SWA 40/61
SAT 1/3	TUR 323/62	SR 2/58, SWA 40/61, BOT 1/68, UGA 47/71, KEN 3/72
SAT 1/4	GHA 14/68	
SAT 1/5	MAL 3/70	BOT 1/68, KEN 3/72
SAT 1/6	RHO 4/72	
SAT 1/7	ANG 9/72	
SAT 1/8	GHA 7/73	NIG 9/72
SAT 1/9	ZAM 25/73	

It is suggested that the proposed system is a more realistic approach than the present sub-type classification, since it is based on the observed characteristics of variation between strains. The main implication is that an antigenic change that occurs to form a new sub-type, does so by a number of small alterations as a gradual process, rather than in a single step. This is reasonable, since the antigenic structure of the virus and the smallness of a change which can alter antigenicity, make the possibilities for continuous minor alterations almost unlimited. It is also supported by evidence of Hyslop (1965) and Hyslop and Fagg (1965). They passaged viruses serially in tissue culture under the selection pressure of antiserum homologous to the starting virus, and through partially immune cattle. Antigenic modification occurred, so that the passaged strains became progressively more different to the starting strains.

Pringle (1969) found that phenotypic mixing between FMDV strains was an infrequent occurrence and argued that as a consequence, any mutation affecting the antigenicity of the virus would be immediately expressed. He suggested that this may in part explain the extensive antigenic variation of FMDV in comparison with, for example poliovirus, which exhibits extensive phenotypic mixing and is antigenically more stable. If this property does in fact allow for an immediate expression of, and selection for, minor antigenic changes, then it would further implicate antigenic drift as the mechanism for intra-type antigenic variation.

In accordance with the proposed new classification, a sub-type can be re-defined as follows: "An FMDV sub-type is an antigenic grouping of closely related strains within a type. All

strains within a sub-type are related to one particular reference strain, such that $R > 25\%$. Reference strains of each sub-type differ from each other to the extent that $R < 25\%$. A strain can be classified into more than one sub-type group according to its relationship to the reference strains."

The role of antiserum quality in the determination of strain differences has not been investigated in detail. however it is recognised that variations in specificity and avidity between different antisera are of considerable importance in this respect.

It is apparent from the results in Sections 2 and 3 that if the 140S antigens of two strains are compared in cross-CF tests, then the values for r for the two antisera are not necessarily equal. Since this inequality is still observed using 12S-adsorbed antisera (Section 2.3), then it cannot be fully explained on the basis of the relative proportions of anti-140S and anti-12S antibody in the sera. However it was shown in this Section (Table 3.6), that the value for r can differ between two antisera of the same strain prepared by different methods. This demonstrates the importance of antiserum quality and the necessity to maintain uniformity in the method of antiserum preparation to minimise this effect. It also suggests the possibility of preparing antisera of even greater specificity. The preliminary results of work currently in progress suggest that it is possible to prepare antisera of greater sub-type specificity, with which small antigenic differences are readily detected even in long-fixation tests.

Stellmann *et al.* (1972 a and b) introduced the concept of dominance into the classification of FMDV strains. They suggested that the antisera of "dominant" strains are more cross-reactive

and proposed a mathematical system for the determination of dominance on the basis of the inequality in values for r.

While the concept of dominance is reasonable and possibly important (for example, in the selection of vaccine strains), it appears for the reasons discussed above, to be dangerous to attribute unequal values of r between two strains, to the effect of dominance. Stellmann et al. provided no information regarding the preparation or quality of their antisera and it is suggested that without adequate characterisation of antisera, the question of dominance cannot be investigated in this way.

3.5 CONCLUSIONS

Strains which had been differentiated by CF, were compared by a guinea pig protection test, kinetic neutralisation and cross-neutralisation tests. It was shown that these tests, which have not been previously applied by the methods described, are all capable of FMDV strain differentiation. Similar differences were found by all methods, which suggests that comparisons made by cross-CF, cross-neutralisation or cross-protection involve measurement of the same antigen/antibody interactions.

The comparison of a large number of strains in cross-CF tests, showed that the range of antigenic variation within a type was greater than had previously been described. Relationships determined between strains, revealed that the present method of sub-type classification is unsatisfactory. A new system was proposed for the sub-type classification of FMDV strains.

SUMMARY

Complement-fixation was one of the earliest techniques developed for quantitative serological studies and it has found extensive application for more than fifty years in the field of FMDV research. The aim of this thesis is to re-examine some of the methods used and the results obtained by earlier workers and to further exploit the application of CF to the study of isolated FMDV antigens.

The methodological studies in Section 1, indicated that some earlier workers (Bradish and Brooksby, 1960; Bradish et al., 1960 a) using CF for FMDV serological studies made certain incorrect assumptions concerning the quantitative aspects of the fixation of complement by FMDV and its antibodies. It was found that in the system described, using antigens which are relatively complex, the fixation of complement follows the principles determined by Mayer et al. (1948) and Osler et al. (1948) using well defined antigen/antibody systems. FMDV strains could be differentiated in CF tests based on these principles, on which the conventional chequerboard titration has been established; i.e. by comparing the titres of antisera when tested with homologous and heterologous antigens at their optimum concentrations.

However, much of the work of Bradish and his associates was confirmed. It was found (Section 2) that the intact virion has no heterotypic activity but cross-reacts between sub-types, while the 12S sub-units, both naturally occurring and prepared by acid-degradation of the virion, have a greater cross-reactivity between sub-types and some heterotypic activity. The non-structural virus infection-associated antigen had virtually no type or sub-

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type specificity. Studies with other isolated antigens revealed that empty capsids were antigenically identical to complete virions, in contradistinction to the findings of workers with other picornaviruses, whose empty capsids are antigenically different, or only partially related to the complete virions. Virus particles treated with trypsin had a reduced sub-type specificity indicating that the polypeptide, VP1, is associated with a highly specific antigenic site.

The use of sub-type specificity studies to compare different viral antigens is an approach which has not been previously applied in FMDV research. However it would appear that it is a sensitive method for examining the relationship between the various structural antigens of the virion.

From the methodological studies of Section 1 and the antigenic analyses of Section 2, it was possible to make a number of observations regarding the application of CF to the type and sub-type identification of FMDV strains. It was suggested that by using antisera of good quality (such as those prepared using purified, inactivated antigens,) a long-fixation CF test could be used for greater sensitivity of antigen detection in typing tests, without the complication of enhanced heterotypic cross-reactions. The use of a short-fixation period was advantageous for sub-type differentiation, as it increased the specificity of the test. A chequerboard titration method, as well as being quantitatively valid, minimises the effect of cross-reactions due to degraded antigen. The method used by earlier workers for sub-type differentiation (Davie, 1964; Darbyshire et al., 1972) was invalid in principle and, in using undiluted crude antigen harvests, predisposed to cross-reactions due to 12S and VIA antigens.

In Section 3, the relevance of CF for FMDV strain differentiation was examined. Quantitative differences between two strains were similar when measured by CF, by cross-protection and by cross-neutralisation tests. It was argued that this was an indication that the reaction measured by CF involves the same antigens which determine immunological specificity in terms of cross-neutralisation and cross-protection.

The results of a large number of cross-fixation tests between FMDV strains of one type, suggested that the present criteria used for the sub-type classification of FMDV strains is unsatisfactory. In particular, the character of antigenic variation, which appears to be a gradual antigenic drift from one sub-type to another, precludes the classification of strains such that each strain falls into only one sub-type group. A new classification system is proposed which is consistent with the observed character of antigenic variation between strains. Only reference strains are uniquely classified, one for each sub-type, while other strains are classified as related strains in one or more sub-type groups.

The importance of antiserum quality was demonstrated, in relation to the measurement of antigenic differences between strains. Antisera differently prepared against the same virus strain had different sub-type specificities. The significance of this was discussed in relation to the determination of "dominant" strains.

Largely as a result of the work described in this thesis, the method for sub-type differentiation of FMDV strains in the World Reference Laboratory has been altered. Comparative studies are in progress to evaluate the modifications proposed for FMDV type differentiation.

APPENDICES

1. Complement-fixation Reagents

Veronal-buffered saline (VBS), used as a diluent for all reagents, was prepared using complement-fixation buffer tablets (Oxoid Ltd., London) giving the following constitution:-

barbitone	0.575 g./l.
sodium chloride	8.5 g./l.
magnesium chloride	0.168 g./l.
calcium chloride	0.028 g./l.
barbitone soluble	0.185 g./l.

Gelatin was added to 0.1% w/v and the pH was approximately 7.2.

The source of complement was serum from fasted, male guinea pigs, which was pooled and twice adsorbed at $0^{\circ}C$ with 3% of washed sheep erythrocytes as recommended by Kabat and Mayer (1961). Complement was stored in small volumes at $-70^{\circ}C$.

Sheep erythrocytes (Wellcome Research Laboratories, Beckenham) stored in Alsever's solution, were washed at least three times in three volumes of VBS or until a clear supernatant was obtained (Kabat and Mayer, 1961). After the final wash, the cells were centrifuged for 10 minutes at 500 g and 1 ml. of packed cells was re-suspended in 50 ml. of VBS. The concentration of erythrocytes was estimated by lysing 1.0 ml. of the suspension with 9.0 ml. of distilled water and measuring the optical density (OD) of the lysate in a Pye Unicam SP500 Series II spectrophotometer at 541 nm. The suspension was then standardised by appropriate dilution to give a lysate with an OD of 0.300 ± 0.005.

Rabbit anti-sheep haemolytic serum (Wellcome) was diluted

1/10 and stored in small volumes at -20°C. Erythrocytes were sensitised by slowly adding an equal volume of haemolytic serum, diluted to contain two minimal haemolytic doses of haemolysin and incubating in a water-bath at 37°C for 30 minutes with occasional mixing. The sensitised erythrocyte suspension was then stored at 4°C prior to use.

2. Preparation of Viruses and Viral Antigens

2 (a) Preparation of infective virus

FMDV strains were obtained from the World Reference Laboratory. Each virus was passaged on cell monolayers of baby hamster kidney (BHK-21, clone 13; Macpherson and Stoker, 1962) in Roux flasks until the virus was capable of destroying the cell sheet within 24 hours after inoculation at a low multiplicity of infection (0.001 to 0.01 pfu./ml.). A monolayer comprising about 10^8 cells was rocked at 37°C during virus growth and maintained with 20 ml. of Eagle's medium supplemented to 10% with tryptose phosphate broth. When the cell sheet was destroyed, the contents of the Roux flask were frozen and thawed and the gross cellular debris removed by low speed centrifugation. The fluid was made up to 50% glycerol and 5% normal bovine serum and stored in small volumes at -70°C as virus seed.

Each strain used to infect guinea pigs was serially passaged by inoculation into the plantar pads until the virus was capable of producing generalised disease within 48 hours. Pads containing infective virus were ground with a pestle and mortar and suspended in 0.04M phosphate buffer pH 7.6 as a 4% w/v suspension of pad tissue. This was clarified by low speed centrifugation, made up to 50% glycerol and stored at -20°C.

2 (b) Preparation of 140S antigen

Virus grown in BHK cell monolayers in Roux flasks was harvested as infected tissue culture fluid, which was held at 4°C for 24 hours prior to low speed centrifugation to remove gross cellular debris. Where an inactivated preparation was required, the harvest was made to 0.05% acetylethyleneimmine (AEI) and held at 26°C for 30 hours. The reaction was then stopped by the addition of 10% of 20% sodium thiosulphate and innocuity verified by two negative serial passages on BHK monolayers. The procedure for virus purification then followed that described by Brown and Cartwright (1963).

To the clarified harvest was added an equal volume of saturated ammonium sulphate in 0.04M phosphate buffer, pH 7.6, at 4°C. After 30 minutes at 4°C the resulting precipitate was deposited by centrifugation at 2000 rpm. (2000g) for 30 minutes in an MSE Mistral centrifuge. It was then drained and suspended in 1/15 of the original volume of 0.04M phosphate buffer and clarified by centrifugation at 10,000 rpm. (12,000g) in the 8 x 50 angle rotor for 30 minutes in an MSE ultracentrifuge. The virus was then sedimented by centrifugation at 30,000 rpm. (108,000g) for 60 minutes in the same rotor.

The pelleted virus was resuspended in 0.04M phosphate buffer to a volume of 1/300 of the harvest, clarified by centrifugation at 12,000g for 15 minutes and made up to 1% w/v sodium dodecyl sulphate (SDS). Sucrose density gradients, 15 to 45% w/v in 0.04M phosphate buffer, were prepared using a two-chamber mixing apparatus and the virus preparation was layered onto the top. Gradients were centrifuged either in the 3 x 25 ml. swinging bucket rotor for 2 hours at 30,000 rpm. (129,000g) or in the

6 x 14 ml. titanium swinging bucket rotor for 90 minutes at 40,000 rpm. (193,000g) in the MSE ultracentrifuge.

After centrifugation, fractions from a gradient were collected by positive pressure displacement through a hole pierced in the base of the centrifuge tube. Fractions containing the virus peak were identified by CF assay, pooled, diluted 1/4 in VBS and stored at 4°C.

2 (c) Preparation of empty capsids

The procedure for isolating empty (RNA-free) capsids was similar to that for the purification of 140S antigen. However, in place of SDS the resuspended pellet preparation was instead made to 1% w/v of Nonidet P40 (BDH Chemicals Ltd. Poole, Dorset) prior to sucrose density gradient centrifugation in the MSE 6 x 14 ml. swinging-bucket rotor. The peak fraction of 75S antigen, detected by CF assay and identified by its position in the gradient, was diluted 1/4 in VBS and stored at 4°C prior to use.

2 (d) Preparation of trypsin-treated virus

Samples containing virus in resuspended pellet material were divided in two. Both parts were incubated at 37°C for 30 minutes, one with 1 mg./ml. of crystallised trypsin (Armour Pharmaceutical Co. Ltd., Eastbourne.) Samples from each were taken for infectivity assay and the rest treated with SDS and purified by sucrose density gradient centrifugation in the MSE 6 x 14 ml. swinging-bucket rotor.

2(e) Preparation of artificial 12S antigen

Peak fractions of 140S antigen from sucrose density gradients were pooled and degraded by the addition of two volumes of 0.05M NaH_2PO_4, producing a final pH of approximately 6.2. The antigen

was then layered onto 15 to 25% sucrose density gradients and centrifuged for 16 hours in the MSE 6 x 14 ml. titanium swinging-bucket rotor at 40,000 rpm. Peak fractions of 12S antigen were identified by CF assay, pooled, diluted 1/4 in VBS and stored in small volumes at -70°C.

2 (f) Preparation of natural 12S and VIA antigens

Natural 12S and VIA antigens were prepared from the supernatant remaining after pelleting the 14OS antigen. The material was re-centrifuged at 30,000 rpm. for 60 minutes in the MSE 8 x 50 angle rotor to further reduce contamination with 14OS antigen. It was then concentrated by ammonium sulphate precipitation, resuspended in a small volume of 0.01M phosphate buffer containing 0.15M NaCl at pH 7.6 (0.01M PBS) and dialysed overnight against this buffer. Material concentrated from the harvest of 20 Roux flask monolayers was then passed through a 15 x 1.5 cm. DEAE cellulose (DE22, Whatman Biochemicals Ltd. Maidstone, Kent) column equilibrated with 0.01M PBS. After collecting 20 2.5 ml. fractions by washing with the equilibrating buffer, the column was then eluted with 320 ml. of a linear gradient of NaCl in 0.01M PBS to 1M NaCl, using a two-chamber mixing apparatus, and 10 ml. fractions collected. Two peaks of complement-fixing activity were identified, one in the sample effluent fractions and one in the NaCl eluate. These were concentrated by ammonium sulphate precipitation and dialysed against 0.04M phosphate buffer. They were then layered onto 15 to 25% sucrose density gradients and centrifuged for 16 hours in the MSE 6 x 14 ml. titanium swinging-bucket rotor at 40,000 rpm. Peaks of complement-fixing activity were identified as 12S and VIA antigens by their relative positions in the gradients (VIA

antigen sediments at approximately 4S), diluted 1/4 in VBS and stored in small volumes at -70°C.

2 (g) Radioisotope labelling of antigens

Radioisotopes were obtained from the Radiochemical Centre, Amersham, Bucks.

Tritiated uridine was used for labelling of RNA. It was incorporated by introducing 100 µCi of ^{3}H uridine into one Roux flask at the time of virus inoculation.

Viral protein was labelled with ^{14}C or ^{35}S. In both instances the virus was grown over a single growth cycle using a multiplicity of infection of 1.0 to 10. 10 µCi of ^{14}C-labelled algal hydrolysate or 200 µCi of ^{35}S-methionine was added to one Roux flask 90 minutes after virus inoculation. Earle's saline or methionine-free Eagle's medium were used for maintenance of the monolayer for ^{14}C and ^{35}S incorporation respectively.

The radioactivity of antigen preparations was determined by drying 50 µl. samples onto discs of Whatman P80 chromatography paper. These were placed into vials containing 2 ml. of scintillant, consisting of 0.1g. POPOP and 4g. PPO per litre of toluene and counting the samples in a Nuclear Chicago Unilux Scintillation Counter.

3. Preparation of Antisera

3 (a) Preparation of antiserum to live virus

Guinea pig antisera to live virus were produced by a method similar to that of Brooksby (1952). Guinea pigs weighing approximately 600 g. were inoculated intradermally in the plantar foot-pads with infective guinea pig adapted virus (Appendix 2 (a)). After three months, they were given a

succession of three hyper-immunising inoculations by the same route on three successive days, then killed and exsanguinated ten days later. The pooled serum was filtered and stored in small volumes at -20°C.

3 (b) Preparation of 140S-specific antiserum

Antisera specific for 140S antigen were prepared in a manner similar to that of Brown and Smale (1970). Live virus antiserum was dialysed overnight against 0.01M phosphate buffer, pH 7.6. Purified 140S antigen was degraded by reduction of the pH to 5.2 by adding 10% v/v of 1M acetic acid and concentrated by vacuum dialysis, against 0.01M phosphate buffer. The dialysed reagents were titrated by CF in tubes to determine the dilutions of antigen and antiserum which were optimally reactive. They were then mixed in a proportion which ensured an antigen excess. The mixture was held at 4°C for 18 hours and then passed through a DEAE-cellulose column, 1.5 cm. x 10 cm., equilibrated and eluted with 0.01M phosphate buffer. Under these conditions, IgM, antigen/antibody complexes and residual excess antigen are retained in the column, while uncomplexed IgG passes through. After passage of the fluid volume of the column, a single peak of high OD was detected. This was collected, inactivated by heating at 56°C for 30 minutes and tested by CF against 140S and natural 12S antigens for specific antibody activity. On verification of anti-140S activity with no detectable anti-12S activity, the antiserum was diluted 1/4 in VBS and stored in small volumes at -20°C.

It should be noted that no attempt was made to remove anti-VIA activity from these antisera; i.e. they were 140S-specific only in their reactivity towards viral capsid components.

3 (c) Preparation of anti-140S serum

Anti-140S guinea pig sera were prepared following the method of Cowan (1968). Purified, AEI-inactivated 140S antigen (Appendix 2 (b)) from the harvest of 20 Roux flask monolayers, was emulsified with incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.) and then re-emulsified with an equal volume of Tween 80 detergent (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) forming a double emulsion. Five guinea pigs were inoculated intramuscularly in the hind-leg and hyper-immunised four weeks later with a similar amount of freshly-prepared 140S antigen, without adjuvant. They were exsanguinated 10 days later and the pooled serum filtered and stored at -20°C.

3 (d) Preparation of anti-12S serum

Artificial 12S antigen was prepared from purified, AEI-inactivated 140S antigen (Appendix 2 (e)). It was emulsified with incomplete Freund's adjuvant and then re-emulsified with an equal volume of Tween 80 detergent. Guinea pigs were inoculated intramuscularly. They were hyper-immunised 36 and 64 days later with similar antigen preparations, without adjuvant, by the same route and exsanguinated after a further 12 days.

4. Method for Routine Complement-fixation Assays

Routine assays for the detection and measurement of the complement-fixing activity of viral antigens were performed in microplates. Details of the reagents used are given in Appendix 1.

Samples were diluted in two-fold series in the plates, using diluting loops. To 25 µl. of antigen were added 25 µl. of a fixed dose of complement ($5C'H_{50}$) and 25 µl. of antiserum, at least four times the concentration of the antiserum titre. Suitable controls were included as appropriate, with complement

doses of 5, 2.5 and 1.25 $C'H_{50}$. Plates were incubated at 37°C for one hour (for detection of 140S, 75S and T-140S antigens) or at 4°C for 18 hours (for detection of 12S and VIA antigens), after which 50 µl. of sensitised erythrocyte suspension was added and the plates incubated for 45 minutes at 37°C. They were then centrifuged for 10 minutes at 600 g. to deposit unlysed cells.

End-points were determined as the highest dilution of antigen which fixed 4 of 5 $C'H_{50}$; i.e. where 50% of the erythrocytes remained unlysed. Where appropriate, interpolation between two dilutions was made by calculating the geometric mean. Results were recorded as the reciprocal of the antigen dilution at the end-point.

5. Method for Polyacrylamide Gel Electrophoresis

The conditions for virus dissociation and electrophoresis of the polypeptides followed those described by Pereira and Skehel (1971). Purified intact or trypsin-treated virus, labelled with ^{35}S-methionine, in 0.04M phosphate buffer, pH 7.2, was mixed with urea, SDS and 2-mercaptoethanol to final concentrations of 5M, 1% and 2% respectively. The mixtures were then heated at 100°C for one minute and, after cooling, a 200 µl. sample was applied to each gel. Gels of 5 ml. volume were prepared in perspex tubes of 6.5 mm. internal diameter. They contained 7.5% acrylamide, 0.2% biscacrylamide, 5M urea, 0.05M sodium phosphate buffer, pH 7.2; 0.01M EDTA, 0.1% SDS and 0.1% TEMED. Polymerisation was catalysed with 0.1% ammonium persulphate. The electrophoresis buffer was 0.05M sodium phosphate, pH 7.2, with 0.1% SDS and 0.01M EDTA. Electrophoresis was carried out for approximately 17 hours at a constant potential of 30 volts.

After electrophoresis, the gels were processed for autoradiography following the method of Russell and Skehel (1972). The gels were sliced longitudinally in an apparatus similar to that of Fairbanks et al.(1965). The slices were washed twice in 7% acetic acid, laid on a porous plate, covered with cellophane and dried under vacuum overnight. The dried gel strips, attached to the cellophane were then used for autoradiography using Kodak X-ray film.

6. Estimation of Virus Infectivity by Plaque Assay

Virus infectivity estimations were carried out by plaque assay using monolayer cultures of IB-RS-2 cells (de Castro, 1964) in 6 cml. Petri plates. The monolayers were grown using a medium of 45% Eagles, 45% lactalbumin yeast hydrolysate (LYH), 10% bovine serum and antibiotics (100 units/ml. each of benzyl-penicillin, neomycin and nystatin). The plates were incubated in an atmosphere of 5% CO_2. The monolayers were confluent after 48 hours and used at that time.

After removal of the growth medium, monolayers were washed once with 5 ml. of phosphate buffered saline (PBS) at room temperature and then inoculated with 200 µl. of the virus samples. A period of 30 minutes at 37°C was allowed for adsorption of virus, after which each monolayer was covered with 5 ml. of overlay, consisting of 47% LYH, 50% Earle's saline, 2% bovine serum and 0.9% agar (Agarose, L'Industrie Biologique Francaise, S.A., France).

The plates were incubated for 30 hours and the monolayers then fixed and stained with 3 ml. of 4% formaldehyde and 10% saturated methylene blue in tap water. After allowing to stand overnight, the overlay was removed and the plaques counted.

For routine infectivity assays, samples were tested by inoculating two plates with each dilution in 10-fold series. The end-point was determined from the mean plaque count of a dilution producing between 10 and 100 plaques on each plate and expressed as plaque-forming units (pfu.) per ml. of undiluted sample.

The plaque assay used for kinetic meutralisation tests is described in Section 3.2 (b).

7. Method for Electrophoretic Mobility Determinations

Electrophoretic mobility determinations were made by sucrose density gradient electrophoresis using apparatus described by Pringle (1969) and modified by Priston (1972).

Cylindrical glass tubes, 60 cm. long and of 1 cm. internal diameter were supported vertically in a water jacket kept at a constant temperature of 10°C. Electrode baths were placed at the top (cathode) and bottom (anode) of the tubes.

Linear sucrose density gradients, 5-40% w/v in 0.01M tris + HCl buffer, pH 8.8, were formed in the tubes, using a two-chamber mixing apparatus. Bovine albumin powder was incorporated into the gradients to 0.1% w/v.

Samples of 140S and T-140S antigens, with phenol red as a marker, were layered onto two columns and electrophoresis carried out for 16 hours at a constant potential of 240 volts and a current of 0.7 to 1.2 ma. per column. Fractions of 1.5 ml. were then collected from the bottom of the column. They were precipitated by adding an equal volume of 20% trichloracetic acid at 4°C and the precipitate collected onto discs of Whatman P80 chromatography paper by suction. These were dried, and their radioactivity determined as described in Appendix 2 (g).

8. The Serum Neutralisation Test

Serum neutralisation tests were carried out in disposable tissue-culture grade microplates (Linbro/Biocult) using IB-RS-2 cells (de Castro, 1964).

The medium used for the dilution of all reagents was 85% Eagles, 10% tryptose phosphate broth, 4% normal bovine serum and 1% w/v of D-glucose. Sera were inactivated at 56°C for 30 minutes and diluted in two-fold steps, either in the plates with diluting loops, or in bottles and added to the wells in 50 µl. volumes with dropping pipettes. A 50µl. drop of virus was added to each well and the plates were incubated for 60 minutes at 37°C in an atmosphere of 5% CO_2. Viruses were titrated in the same test, using $\log_{10}$ 1.0 intervals with eight replicates. Reference sera were also included where appropriate, again using eight replicates. After incubation cells were added in a 50 µl. drop at a concentration of 1.5×10^6/ml. The plates were then sealed with adhesive tape and incubated at 37°C for 48 hours.

Tests were read by examining the monolayers microscopically or by fixing with formalin and staining with methylene blue. The virus was considered neutralised if more than half of the monolayer in a well was intact. Antibody titres were determined by the method of Kärber (1931) and expressed as the reciprocal of the highest dilution of serum which resulted in neutralisation of the virus.

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